Bone Marrow Precursors of Nonobese Diabetic Mice Develop into Defective Macrophage-Like Dendritic Cells In Vitro[1]

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The NOD mouse spontaneously develops autoimmune diabetes. Dendritic cells (DC) play a crucial role in the autoimmune response. Previous studies have reported a defective DC generation in vitro from the NOD mouse bone marrow (BM), but a deviated development of myeloid precursors into non-DC in response to GM-CSF was not considered. In this study, we demonstrate several abnormalities during myeloid differentiation of NOD BM precursors using GM-CSF in vitro. 1) We found reduced proliferation and increased cell death in NOD cultures, which explain the previously reported low yield of DC progeny in NOD. Cell yield in NOR cultures was normal. 2) In a detailed analysis GM-CSF-stimulated cultures, we observed in both NOD and NOR mice an increased frequency of macrophages, identified as $CD11c^+/MHCII^-$ cells with typical macrophage morphology, phenotype, and acid phosphatase activity. This points to a preferential maturation of BM precursors into macrophages in mice with the NOD background. 3) The few $CD11c^+/MHCII^{high}$ cells that we obtained from NOD and NOR cultures, which resembled prototypic mature DC, appeared to be defective in stimulating allogeneic T cells. These DC had also strong acid phosphatase activity and elevated expression of monocyte/macrophage markers. In conclusion, in this study we describe a deviated development of myeloid BM precursors of NOD and NOR mice into macrophages and macrophage-like DC in vitro. Potentially, these anomalies contribute to the dysfunctional regulation of tolerance in NOD mice yet are insufficient to induce autoimmune diabetes because they occurred partly in NOR mice. *The Journal of Immunology*, **2004, 173: 4342–4351.**

Insulin-dependent diabetes mellitus (IDDM)[3] or type I diabetes develops as a result of an autoimmune destruction of the β cells in the islets of Langerhans (1). To investigate the pathogenesis of IDDM, animal models such as the nonobese diabetic (NOD) mouse and the biobreeding rat have proved to be valuable tools. These animals develop IDDM spontaneously. In NOD mice, the sequence of events that occurs during this process closely resembles the disease development in humans (2).

Dendritic cells (DC) and macrophages play a pivotal role in the initiation and progression of the autoimmune response and the final destruction of the β cells. In the pancreas of neonatal NOD mice, elevated numbers of DC and macrophages are present (3). Furthermore, an accumulation of DC and macrophages at around 4 wk of age marks the initiation of the autoimmune insulitis before the infiltration of lymphocytes (4, 5). The final β cell destruction is thought to occur by the activity of both lymphocytes and macrophages (4, 6). Together, these findings underline the important roles of DC and macrophages in the different stages of the inflammatory process in the pancreas.

As APC, DC play a prominent role in both central and peripheral tolerance. Therefore, deficient induction and maintenance of tolerance by aberrant DC would be key to the initiation of autoimmunity (7). According to such a view, a hampered negative selection by aberrant thymus DC leads to the incomplete deletion of autoreactive T cells. In addition, abnormal DC in the periphery might not stimulate regulatory T cells sufficiently, thus contributing to a failing control of the autodestructive process in the target organ. These notions have led to an extensive investigation of a putative deficient function of DC in type I diabetes. In both humans and biobreeding BB-DP rats, a functional deficiency of DC has been observed. Fewer DC, an immature phenotype, and impaired stimulatory capacity have been found in both human and rat DC (8–10). In contrast to these studies, ex vivo isolated NOD mouse DC from spleen and lymph nodes demonstrated only minor phenotypic and functional aberrations (11, 12).

In marked contrast to the reports on ex vivo DC isolated from prediabetic NOD mice, in vitro studies show significant abnormalities in DC yield and development when NOD DC are generated from bone marrow (BM) precursors (12–16). Studies where NOD BM precursors are stimulated with GM-CSF alone show generation of low numbers of DC that display an immature phenotype and a poor T cell-stimulatory capacity (13, 14, 17). However, in the latter reports, the authors describe the phenotypic and functional anomalies of the in vitro generated NOD myeloid DC, without assessing a possibly deviated development of NOD myeloid precursors into non-DC. Because GM-CSF is an important inflammatory cytokine that influences the function and development of DC but also of other myeloid cell types (18), we decided to investigate the in vitro effects of GM-CSF on the generation of NOD BM cells in detail, with emphasis on the heterogeneity of the developing cell types.

Our study shows several anomalies in myeloid DC differentiation and maturation in NOD and NOR (as compared with C57BL and BALB/c) cultures that lead to a strongly decreased generation of prototypic and mature DC. Furthermore, phenotypically mature DC, when separated, appear to have various macrophage characteristics and a reduced T cell-stimulatory capacity, despite normal

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[3] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; DC, dendritic cells; BM, bone marrow; AP, acid phosphatase; Flt3-L, Flt3 ligand; 7-AAD, 7-aminoactinomycin D; PI, propidium iodide; TfR, transferrin receptor.

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expression levels of MHC and costimulatory molecules. The aberrant maturation of myeloid DC also occurs in Flt3 ligand (Flt3-L)-stimulated BM cultures, which points toward an intrinsic defect in myeloid DC development in mice with the NOD background.

Materials and Methods

Mice

Female NOD/Ltj (prediabetic), C57BL/6J, BALB/c, NOR/Lt, and C3Heb/Fej mice 5–10 wk of age were used in this study. NOD/Ltj and C3Heb/Fej mice were bred at the animal facility of the Erasmus MC (Rotterdam, The Netherlands). C57BL, BALB/c, and female NOR mice were obtained from Harlan (Horst, The Netherlands). Female C3HeB/FeJ mice were used as a source of allogeneic responder T cells. Animals were specific pathogen free and had free access to food and water in the animal care facility at the Erasmus MC Rotterdam under the institutional guidelines for usage of experimental animals.

mAbs and conjugates

mAbs were used for cell sorting, flow cytometric analysis and evaluation of T cell activation in the MLR. Undiluted culture supernatants of the hybridomas 2.4G2 (anti-FcRγII/III-CD16/32), ER-MP21 (anti-transferrin R-CD71), as well as unlabeled Ab against the scavenger R type I (SR-AI/II-2F8; kindly provided by Dr. S. Gordon) were detected by FITC- or R-PE-labeled goat anti-rat IgG (mouse-absorbed; GαRa-FITC or GαRa-PE) purchased from Caltag Laboratories (San Francisco, CA). Directly labeled 53-6.7^{FITC} (anti-CD8α), RM4-5^{PE} (anti-CD4), and HL3^{PE} (anti-CD11c) were purchased from BD Biosciences (San Diego, CA). Biotinylated ER-TR3 (anti-MHC class II; I-A) was kindly provided by BMA Biomedicals (Augst, Switzerland) and 10-2.16 (anti-MHC class II for NOD; I-A^{g7}) was produced, purified, and biotinylated in our laboratory. These Abs were detected by R-PE (Caltag) or allophycocyanin (BD Biosciences)-conjugated streptavidin.

rGM-CSF- and Flt3-L-stimulated BM cultures

Single-cell suspensions of BM, isolated from femora and tibiae, were prepared as described previously (19). Total BM cells were cultured in RPMI 1640 (without HEPES; BioWhittaker, Walkersville, MD) supplemented with 10% FCS (heat-inactivated; 0.2-μm pore size filtered), 60 μg/ml penicillin, and 100 μg/ml streptomycin (further referred to as culture medium). Growth factors were used according to published protocols for DC generation (20, 21). In GM-CSF-stimulated cultures, a final concentration of 20 ng/ml recombinant GM-CSF (Biosource International, Camarillo, CA) was used, whereas 100 ng/ml recombinant mouse Flt3-L (R&D Systems Europe, Abingdon, U.K.) was used in Flt3-L-stimulated cultures. Cells were cultured at 37°C, 7% CO_2 for various periods of time, as indicated, up to 10 days.

Cell suspensions

Cultured BM-derived cells were isolated from culture dishes by vigorous pipeting to collect all cells. Cells were washed and kept until further use in culture medium containing no additional growth factors. When used for phenotypic analysis, cultured cells were washed with PBS supplemented with 0.5% (v/v) BSA (Organon Teknika, Boxtel, The Netherlands) and 20 mM sodium azide. Single-cell suspensions of spleen and lymph nodes were prepared and used for T cell isolation as described (11).

Cell sorting and flow cytometric analysis

For cell sorting, BM-derived cultured cells were labeled with two (anti-CD11c and anti-MHC class II) mAbs as described previously (22). Before sorting (FACSVantage; BD Biosciences), cell suspensions were filtered over a 30-μm pore size sieve (Polymon PES; Kabel, Amsterdam, The Netherlands) to avoid clogging of the nozzle. After sorting, the purity of the cell suspensions was checked by rerunning sorted samples, and purity exceeded 95%. Suspensions were kept at 4°C throughout the staining and sorting procedure. Sorted cells were counted in a Bürker hemocytometer.

For phenotypic analysis by flow cytometry, samples of cultured cells ($\geq 2 \times 10^5$ cells) were aliquoted into 96-microwell plates (round-bottom; Nunc, Roskilde, Denmark) and labeled with Abs as described before (22). For dead cell exclusion, labeling with 7-aminoactinomycin D (7-AAD; Molecular Probes Europe, Leiden, The Netherlands) was used before the measurement (23). Events ($3–5 \times 10^4$) were scored using a FACSCalibur flow cytometer and analyzed by CellQuest software (BD Biosciences).

Analysis of the cell cycle and apoptosis

Apoptosis and cell cycle characteristics were determined by using a propidium iodide (PI) staining as previously described (24, 25). In short, 1×10^6 cells were fixed in ice-cold ethanol for >2 h. Subsequently, cells were incubated at room temperature for 30 min in PBS containing 0.02 mg/ml PI, 0.1% v/v Triton X-100, and 0.2 mg/ml RNase; left overnight at 4°C; and analyzed with FACSCalibur. Doublet cells were excluded from the analysis by measuring peak area and width. The number of proliferating cells was determined by gating the region of the DNA histogram that contained cells in G_1-G_0, S, and G_2-M phases of the cell cycle. The percentage of apoptotic cells was determined by gating the sub-G_1 peak in the same DNA histogram.

Cytochemistry

The acid phosphatase (AP) activity of the various culture-derived populations was determined using cytospins of sorted cells prepared on a cytospin apparatus (Nordic Immunological Laboratories, Tilberg, The Netherlands). Cytospins were air dried and used for the AP staining according to the method of Katayama et al. (26), using naphthol AS-BI phosphate as a substrate and a hexazotized pararosanilin as a coupling agent (37°C, 30 min). Slides were counterstained with hematoxylin. Preparations were mounted in DePex mounting medium (Gurr, BDH, Poole, U.K.).

Stained cytospins were quantified by counting a minimum of 300 cells/sample. For each mouse strain, four independent samples per experiment were counted using the following characterization. AP stains the lysosomal compartment (27), and on the basis of the staining intensity and distribution we classified four different staining patterns: negative cells (granulocytes and myeloid precursors); weak cytoplasmic staining (cells with several small positive lysosomes, immature DC (28)); strong cytoplasmic staining (cells with strong AP activity in numerous lysosomes spread throughout the cytoplasm, typical for macrophages (29); and single-dot staining (mature DC with a dot-like AP-active region located in the cell center (28).

MLR assay

The capacity of in vitro generated BM-derived cells to activate allogeneic T cells was measured as previously described (19). Briefly, stimulator cells (cultured DC) and responder T cells, isolated from lymph nodes, were resuspended in RPMI 1640 supplemented with 25 mM HEPES, 10% FCS (heat inactivated; 0.2-μm pore filtered), 60 μg/ml penicillin, 100 μg/ml streptomycin, 20 mg/L sodium pyruvate, and 50 μM 2-ME (further referred to as MLR medium). T cells were resuspended at a concentration of 10^6/ml, and the concentration of stimulator cells varied depending on the desired stimulator-responder cell ratio. The cells (100 μl of each cell suspension) were incubated in round-bottom 96-well plates for 4 days at 37°C in 7% CO_2. Stimulator and responder cells, incubated separately in MLR medium, were used as negative controls. Mitogenic stimulation of T cells by Con A (final concentration, 1.25 μg/ml) (Sigma-Aldrich, St. Louis, MO) was used as a positive control.

We used induced expression of the transferrin receptor (TfR/CD71) as a measure for T cell proliferation in the MLR, because the iron uptake through transferrin by stimulated cells has been shown to be a necessary requirement for their proliferation (30). Control experiments demonstrated that results obtained by [^{3}H]thymidine incorporation correlated directly with the results obtained with our method using the CD71/TfR expression detection (Ref. 19) and our unpublished data). In addition, we compared the kinetics of the CD71/TfR expression with other activation markers (CD25, CD69) on allogeneic T cells stimulated by in vitro generated NOD DC. The expression pattern of CD71/TfR was similar to the expression of CD25 or CD69; therefore, we chose to use CD71/TfR expression analysis as a representative readout of the T cell stimulation in the allogeneic MLR.

After 4 days of coculture, cells were harvested and analyzed by flow cytometry. Triple labeling of cells with anti-CD4, anti-CD8, and anti-CD71/TfR Abs was performed, and 1.5×10^4 events were acquired within the live-cell gate. Data was analyzed using CellQuest analysis software. The percentage of CD71/TfR-positive cells was determined within the $CD4^+$ and $CD8^+$ population.

Statistical analysis

Statistical analyses were done by a paired two-tailed Student *t* test using the SPSS software package to determine differences within the means of the sample groups. Results are presented as the mean ± SEM, unless otherwise indicated.

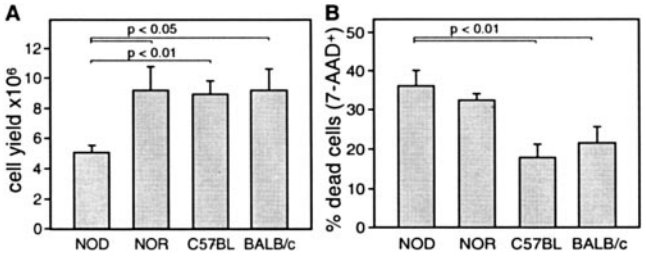

FIGURE 1. Low cell yield and increased cell death in NOD GM-CSF-stimulated BM cultures. Total BM was stimulated with GM-CSF for 7 days in vitro. *A*, Total yield of viable cells from NOD cultures was significantly lower than from NOR, C57BL, or BALB/c cultures. Data represent average values ± SEM derived from 2 (of 10 performed) experiments in which the BM from all 4 strains was cultured simultaneously. $p < 0.01$ NOD vs C57BL and $p < 0.05$ NOD vs NOR and BALB/c. *B*, The frequency of dead (7-AAD^+) cells at day 7 of culture in NOD is significantly higher than in C57BL and BALB/c but not higher than in NOR cultures. Values are the average ± SEM derived from all 10 experiments. $p < 0.01$ NOD vs C57BL and BALB/c.

Results

Low yield of DC in GM-CSF-stimulated NOD BM cultures is caused by reduced proliferation and increased apoptosis

The capacity of NOD BM precursors to generate DC in vitro has been studied previously, and several groups reported a low yield of cells in NOD cultures stimulated with GM-CSF alone (12–14, 17). Similarly, we found that NOD cultures on average contained a significantly lower number of cells than the cultures from C57BL and BALB/c mice ($p < 0.01$). (Fig. 1*A*). This appeared not to be caused by a decreased frequency of GM-CSF-responsive precursors in NOD BM as determined by limiting dilution analysis (data not shown). Interestingly, the yield of cells in NOR cultures was similar to those in C57BL and BALB/c mice (Fig. 1*A*).

To study the events causing the low yield of cells in NOD cultures stimulated with GM-CSF, we followed kinetic changes in the number of proliferating vs apoptotic cells at different time points of the culture (days 2, 5, and 7) by labeling the nuclear DNA content with PI. Proliferation appeared to be quite constant during the culture, but the different mouse strains had different proliferation rates (Table I). Of three mouse strains tested, BM cells from the C57BL mouse showed the highest proliferation rate, significantly higher than the NOD mouse at days 5 and 7 ($p < 0.01$). The proliferation rate in the BALB/c culture was on average also higher than in the NOD culture, but this difference did not reach statistical significance, except for day 5 of the culture ($p < 0.05$). Therefore, increased proliferation partly contributed to the higher cell number in the culture of the control strains.

Using the same DNA staining, we could determine apoptosis in the cultures (cells with the nuclear content <2n) and found the highest percentage of dead cells at the earliest time point (day 2) in all cultures (Table I). This was due to apoptosis of many BM cells unable to respond and survive when stimulated by GM-CSF (mature granulocytes or cells that belong to the erythroid and lymphoid lineage). At later time points, the number of apoptotic cells decreased in the C57BL and BALB/c cultures, which was not the case in NOD cultures. Therefore, the observed higher cell death in NOD cultures additionally contributed to the lower yield at the end of the culture period. NOR mice were not tested in this assay.

A more sensitive method to quantify dead cells at the end of the culture was the uptake of 7-AAD shortly before the sample acquisition. Using this method, we found an even more pronounced difference in the frequency of dead or dying cells in NOD cultures. Significantly higher percentages were found in NOD cultures than in C57BL and BALB/c cultures ($p < 0.01$; Fig. 1*B*). NOR mice were also tested, and the percentage of dead cells was higher in NOR than in C57BL and BALB/c cultures. Therefore, NOR mice show aberrance regarding cell apoptosis similar to NOD, yet cell yield is normal and comparable with those of C57BL and BALB/c mice (Fig. 1).

Diverged differentiation of BM cells to macrophages in NOD and NOR cultures

To investigate the possibility that NOD BM precursors demonstrated reduced proliferation and augmented apoptosis due to an aberrantly fast maturation, we analyzed BM cultures at different time points by double labeling for CD11c and MHC class II. From the expression pattern of these two markers, three DC developmental stages can be defined in normal BM cultures (Ref. 20 and morphological analysis below): granulocytes and myeloid precursors ($CD11c^-MHCII^-$), immature DC ($CD11c^+MHCII^{medium}$), and mature DC ($CD11c^+MHCII^{high}$). These three subsets were phenotypically easily discerned in cultures of C57BL mice (Fig. 2) as well as other control mouse strains (data not shown). In addition, a small population of $CD11c^+MHCII^-$ cells was found in all BM cultures. These cells are considered to be monocytes/macrophages (M. Lutz, unpublished observations; see also below) and they measured <10% of viable cells in cultures of control mice.

In comparison to C57BL, the developing NOD BM cultures showed a strikingly different cellular composition over time. At days 2 and 5 of culture, reduced frequency of $CD11c^+$ cells was found in NOD cultures (6% in NOD vs 13% in C57BL and 24% in NOD vs 46% in C57BL, for days 2 and 5, respectively; Fig. 2). This difference was less pronounced at day 7 of culture (55% vs 64% $CD11c^+$ cells in NOD and C57BL, respectively). However, in marked contrast to the C57BL cultures, at day 7 the majority of $CD11c^+$ cells in NOD culture were MHC class II^-, and a significantly reduced proportion of cells expressed MHC class II molecules (17% in NOD cultures as compared with 58% $CD11c^+MHCII^+$ cells in C57BL). From these data, we concluded that in response to GM-CSF, NOD BM precursors do not show fast

Table I. *Proliferation and apoptosis analysis of BM cultures at different time points*

	Proliferation[a]			Apoptosis[b]		
Day of Culture	NOD	C57BL	BALB/c	NOD	C57BL	BALB/c
2	18.1 ± 3.8	26.5 ± 3.7	19.9 ± 4.5	25.8 ± 7	20.1 ± 4.9	23.1 ± 9.7
5	11.4 ± 0.5	23.2 ± 1.7[c]	19.0 ± 1.4[d]	11.4 ± 3.2	4.7 ± 1.6	10.5 ± 2.1
7	15.0 ± 1	24.6 ± 2.1[c]	19.7 ± 1.9	9.4 ± 2.2	3.1 ± 0.3	6.0 ± 1.9

[a] Average frequency (±SEM) of cells with nuclear content >2n.
[b] Average frequency (±SEM) of cells with nuclear content <2n.
[c] $p < 0.01$ NOD vs C57BL.
[d] $p < 0.05$ NOD vs BALB/c.

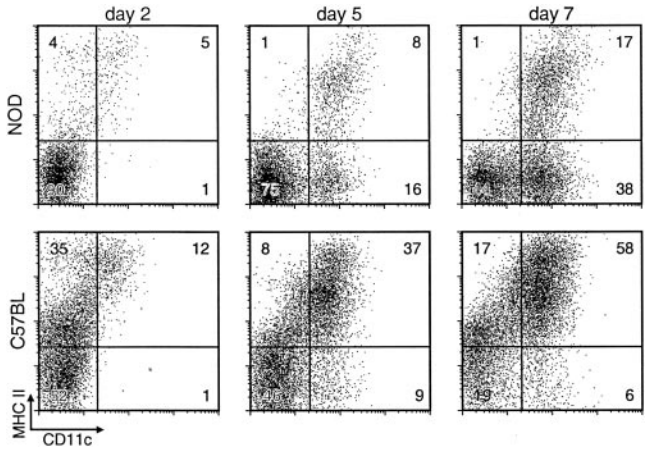

FIGURE 2. Deviated development of NOD DC in vitro. GM-CSF-stimulated cells from NOD and control (C57BL) were harvested at different time points and labeled with CD11c and MHC class II Abs. After gating out dead cells (7-AAD$^+$), at days 2 and 5 of culture, a reduced percentage of CD11c$^+$ cells was present in NOD culture. Up-regulation of MHC class II in NOD cultures was also diminished, leading to the development of fewer mature DC (CD11c$^+$MHCII^{high}) in culture. In addition, an increased percentage of CD11c$^+$MHCII$^-$ cells developed. Numbers indicate the percentage of cells in the quadrants. Dot plots are representative of five separate experiments with similar results.

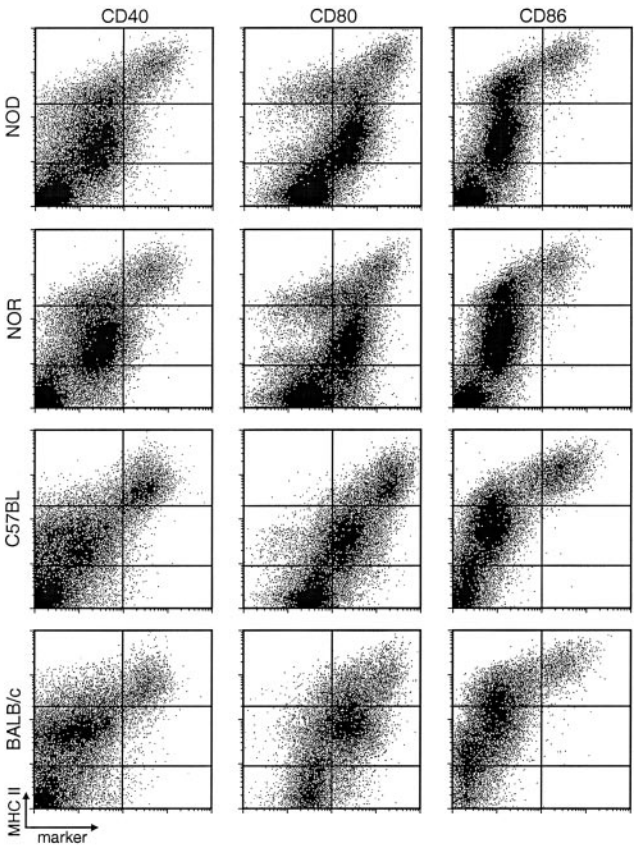

FIGURE 3. Few mature DC (CD11c$^+$MHCII^{high}) develop in NOD and NOR cultures, but they show normal expression of costimulatory molecules. NOD, NOR, C57BL, and BALB/c cells obtained from 7-day GM-CSF-stimulated BM cultures were double-labeled with MHCII and CD80, CD86, or CD40 mAbs. Dot plots show all culture-derived cells, after gating out dead cells (7-AAD$^+$). Representative dot plots are shown for each mouse strain from the same experiment. In total three to seven independent cultures per mouse strain have been performed with similar results.

maturation to myeloid DC but, in contrast, a deviated maturation into CD11c$^+$MHCII$^-$ cells.

Cells from BM cultures of NOR mice displayed a CD11cMHCII staining pattern similar to that of NOD cultures. At day 7 of culture, NOR cultures contained 27% myeloid precursors (CD11c$^-$MHCII$^-$), 22% monocytes/macrophages (CD11c$^+$MHCII$^-$), 29% immature DC (CD11c$^+$MHCII^{medium}), and 21% DC with mature phenotype (CD11c$^+$MHCII^{high}).

Further, we analyzed the expression of the costimulatory molecules CD40, CD80, and CD86 by the different subsets, distinguished on the basis of MHC class II expression (Fig. 3). Expression of costimulatory molecules by cells derived from mice of the NOD background (NOD and NOR) was similar to those of the control cultures. Mature DC (CD11c$^+$MHCII^{high}) from NOD and NOR cultures, although present at a reduced frequency, expressed similar levels of CD40, CD80, and CD86 compared with the C57BL and BALB/c. Immature, MHC class II^{medium} DC in all mouse strains were CD80^{low}, CD86^{low}, and CD40$^-$, whereas MHCII$^-$ cells were negative for all three markers showing again that these cells in NOD and NOR cultures were not DC that merely failed to up-regulate MHC class II molecules.

Taken together, subset analysis of BM cultures showed that NOD and NOR precursors have a decreased potential to develop into DC (MHCII$^+$CD11c$^+$) when stimulated by GM-CSF. However, the relatively few immature (MHCII^{low}CD11c$^+$) and mature (MHCII^{high}CD11c$^+$) DC that develop in these cultures express normal levels of costimulatory molecules on their cell surface.

Poor stimulatory capacity of DC generated in GM-CSF cultures from NOD and NOR mice

In addition to the different frequency of mature DC, BM cultures showed different strain-specific capacities to stimulate T cells in the allogeneic MLR (Fig. 4). In two independent experiments, NOD as well as NOR DC failed to stimulate significant expression of CD71/TfR on the surface of allogeneic (C3H) T cells upon 4 days of coculture (Fig. 4, *A* and *B*). In control experiments, we excluded the low response of T cells due to the responder mouse strain as a possible cause for the low MLR outcome. In addition, poorer MLR capacity of stimulators from NOD and NOR cultures did not originate from the MHC diversity with the controls, because DC of the congenic NOD^{H2b} mice with the MHC region derived from C57BL strain showed the same NOD-like low stimulation capacity (data not shown). Taken together, GM-CSF-generated DC from the mice that share the NOD background also share a poor stimulation capacity.

The poor stimulation capacity of NOD and NOR DC could not be overcome by an additional maturation stimulus. Unlike DC from C57BL and BALB/c cultures, NOD and NOR DC failed to increase their T cell activation potential when stimulated overnight with 100 ng/ml LPS (Fig. 4*C*). However, the quality of T cells stimulated by any of the strains was similar with respect to the cytokine production; they produced significant amounts of IFN-γ and little IL-4 or IL-10 and therefore were Th1-like. When calculated per cell, all T cells produced similar levels of cytokines irrespective of the origin of DC by which they were stimulated (data not shown).

The very low frequency of mature DC in NOD cultures observed in phenotypic analysis might have been the reason for the poor performance of the whole population in the MLR. Therefore, BM subsets were purified by cell sorting and cocultured with allogeneic T cells (Fig. 5). Although devoid of potential inhibitory cells and present in the same numbers as in the control, purified

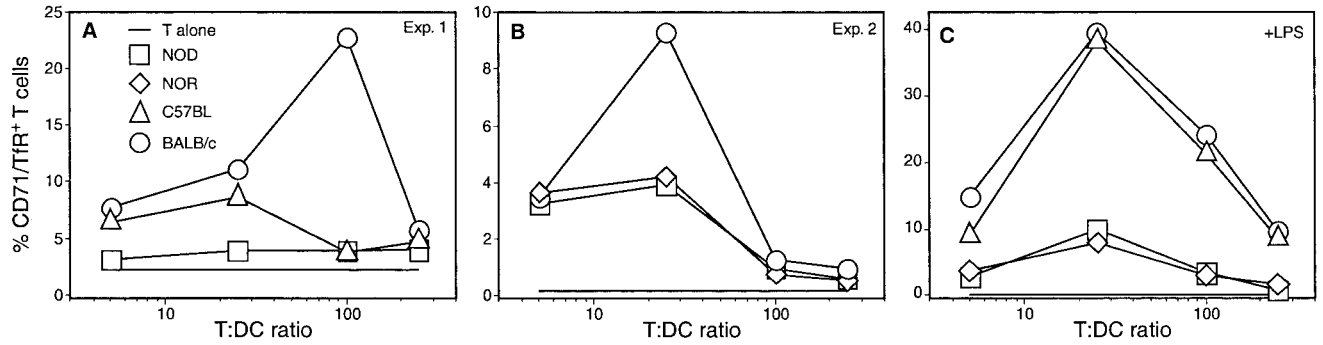

FIGURE 4. Poor stimulatory capacity of total culture-derived cells generated from NOD and NOR mice. *A* and *B*, Total BM culture-derived cells were cocultured for 4 days with allogeneic T cells from C3HeB/Fej mice in different APC-T cell ratios. The capacity of total culture cells from NOD mice is compared with those of C57BL or BALB/c mice in experiment 1 and with NOR or BALB/c mice in experiment 2. In contrast to the other two mouse strains, NOD and NOR cells exhibited similar poor stimulatory capacity. *C*, LPS stimulation increased the stimulation potential of BM cells from all four strains. However, stimulated NOD and NOR cells exhibited again a much lower stimulatory capacity than C57BL or BALB/c cells. Data present the percentage of T cells ($CD4^+$ and $CD8^+$) that express CD71/TfR as indication of their productive stimulation by APC. A representative of at least three independent MLR cultures for each mouse strain is shown.

mature NOD DC ($CD11c^+MHCII^{high}$) failed to stimulate T cells to a significant level when compared with C57BL mature DC (Fig. 5*E*). The percentage of T cells that had up-regulated CD71/TfR when stimulated by NOD mature DC (Fig. 5*E*) was rather comparable with the proportion induced by the immature DC or monocytes/macrophages from C57BL culture (Fig. 5, *C* or *D*). Other purified subpopulations from NOD cultures also exhibited lower stimulatory capacity than the corresponding C57BL subpopulations.

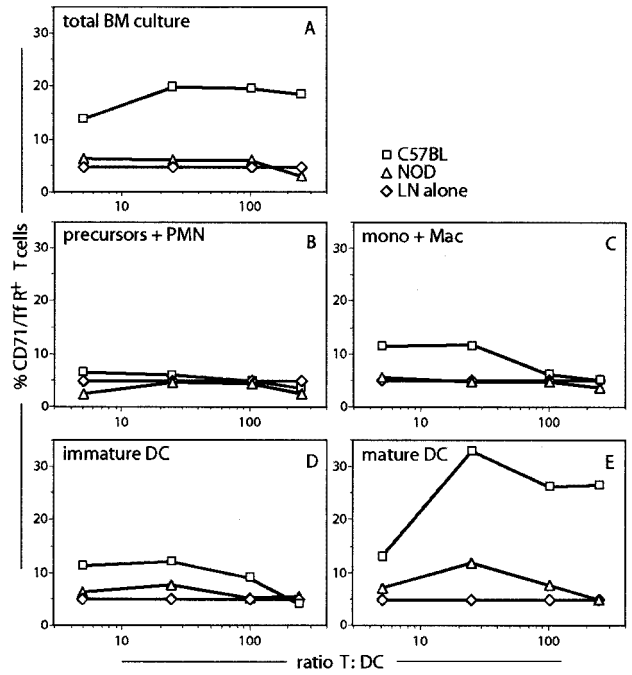

FIGURE 5. Poor capacity of sorted mature DC from NOD BM cultures to stimulate T cells. Sorted cells from 7-day cultures were cocultured with allogeneic T cells at different APC-T cell ratios. Data are presented as percentage of T cells that express CD71/TfR. Unseparated NOD culture-derived cells were virtually unable to stimulate T cell proliferation, in contrast to their C57BL counterparts. As expected, purified mature DC ($CD11c^+MHCII^{high}$) from the C57BL culture displayed the highest stimulating capacity, whereas NOD mature DC were poor stimulators even when purified. Data are representative of two experiments performed independently, with similar results. Mac, Macrophages; mono, monocytes; LN, lymph nodes.

Morphology of mature DC in NOD cultures is similar to the immature DC from the C57BL

To characterize further the four cell subsets identified in flow cytometric analysis (see before), we investigated the morphology of cells separated by cell sorting from day 7 BM culture on the basis of their differential expression of CD11c and MHC class II molecules (Fig. 6*A*). As shown in Fig. 6*B*, the $CD11c^-MHCII^-$ (R1) population contained myeloid precursors (slightly indented large nucleus with more basophilic cytoplasm) and neutrophilic granulocytes (typical segmented nucleus) in both NOD and C57BL cultures. The $CD11c^+MHCII^-$ (R2) fractions, abundantly present only in NOD, contained morphologically distinguishable monocyte/macrophage-like cells in both cultures (asymmetrically positioned oval or kidney-shaped nucleus and a high cytoplasm-nucleus ratio). The $CD11c^+/MHCII^{medium}$ (R3) cells derived from C57BL BM resembled immature DC (relatively large cells with an oval nucleus on one cell side and a large cytoplasm, more regular in shape) which formed relatively large clusters. In contrast, cells of the same $CD11c^+MHCII^{medium}$ population in NOD cultures hardly formed any clusters. Finally, $CD11c^+MHCII^{high}$ (R4) cells from C57BL cultures had an irregular shape and a number of dendritic protrusions, thus resembling mature DC that mostly occurred as single cells or formed small clusters. The morphology of phenotypically mature DC separated from NOD cultures did not differ significantly from their C57BL counterparts, but they formed large clusters comparable with those of immature C57BL DC.

Mature NOD and NOR myeloid DC express higher levels of markers typical for macrophages

The defective T cell stimulatory capacity and large cluster formation of separated mature DC from NOD BM cultures prompted us to investigate their phenotype further. Because NOD BM precursors seem to develop more readily into monocytes/macrophages ($CD11c^+MHCII^-$) in vitro, we focused on macrophage markers in particular. As shown in Fig. 7, mature DC ($CD11c^+MHCII^{high}$ cells) from C57BL cultures express very low to negative levels of FcRγ II/III, scavenger R, F4/80, and Gr-1. In contrast, much higher levels of these markers were found on mature NOD DC. When quantified from other mouse strains (Table II), tested markers were higher on mature DC from NOD mice than on both C57BL and BALB/c mature DC. Interestingly, scavenger R and F4/80 were expressed on the NOR mature DC in a

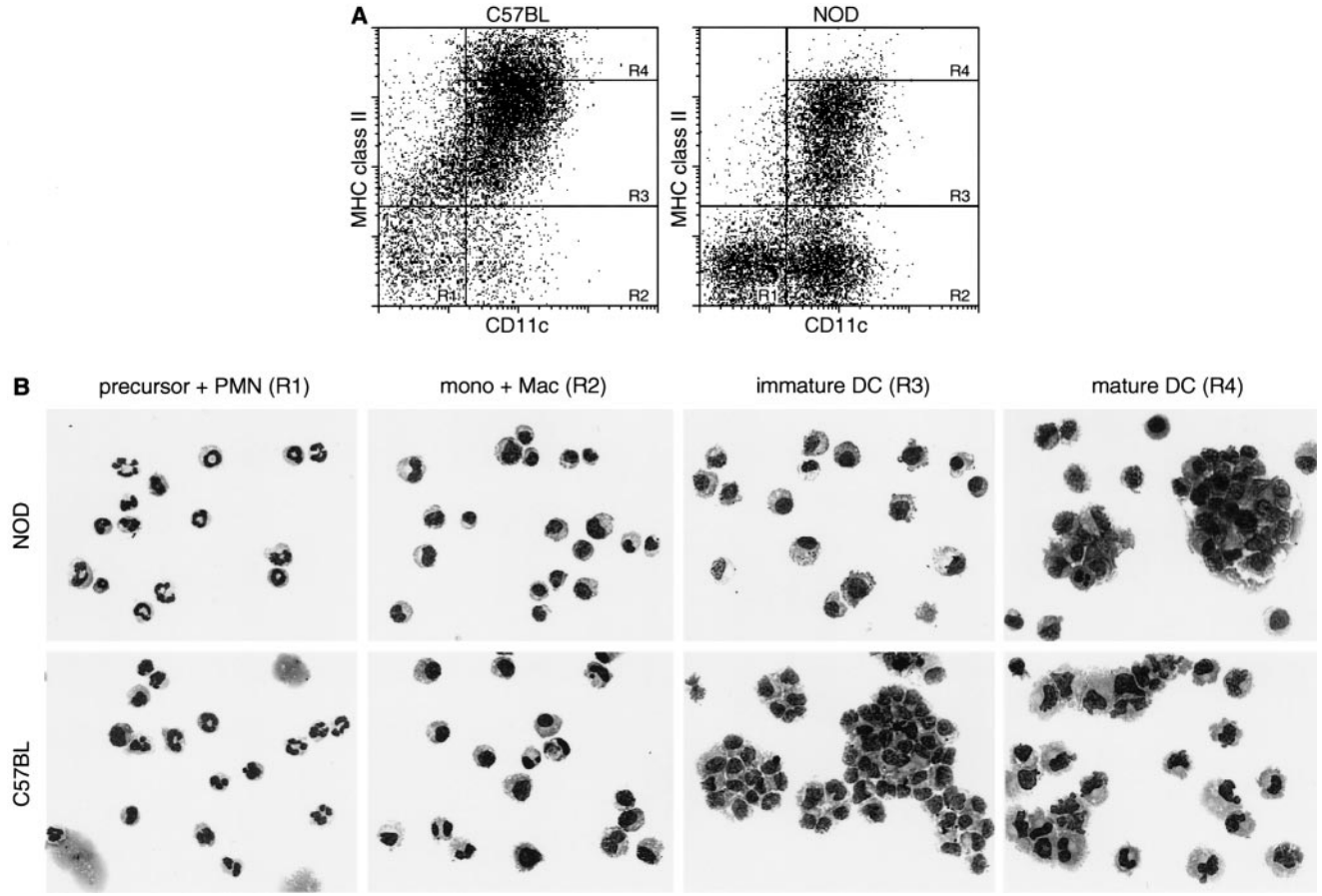

FIGURE 6. Morphological analysis of cells in GM-CSF-stimulated culture shows similar subpopulations in NOD and control cultures. A. Four subpopulations of cultured cells can be defined with MHCII and CD11c: (R1) $CD11c^{-}/MHCII^{-}$; (R2) $CD11c^{+}/MHCII^{-}$; (R3) $CD11c^{+}/MHCII^{med}$ and (R4) $CD11c^{+}/MHCII^{high}$. Represented gates are used for cell sorting. B. Sorted cells are morphologically identified as follows: R1-myeloid precursors and neutrophils; R2- monocytes (mono)/macrophages (Mac); R3, immature DC; R4, mature DC (more details in *Results*). Magnification, ×126.

manner similar to that of the NOD, whereas FcRγ II/III and Gr-1 values were intermediate between the NOD and the other two strains.

Mature DC from NOD cultures contain an abnormally high percentage of macrophage-like cells with a strong lysosomal activity

To assess the putative macrophage-like nature of mature DC generated in NOD cultures, we performed cytochemical staining of AP (for categorization/scoring, see *Materials and Methods*) in sorted cell populations (Fig. 8*A*). Initial examination revealed that the AP pattern of the sorted populations from the C57BL cultures overlapped to a great extent to their phenotype. This was not the case in sorted cells from NOD cultures. When quantified (Fig. 8*B*), the R3 population from NOD cultures with the phenotype of immature DC also included a significant percentage of cells with a lysosomal activity typical of macrophages (strong cytoplasmic staining) (49% for NOD vs 8.3% for C57BL). An even more pronounced difference between NOD and C57BL existed in the sorted $CD11c^{+}MHCII^{high}$ population (R4) of phenotypically mature DC. This population isolated from NOD cultures contained few cells with the staining pattern typical of mature DC (single-dot staining; 3.3% for NOD vs 83% for C57BL) and a remarkably high percentage of cells with staining like that of macrophages (67.3% for NOD vs 7.7% for C57BL).

To investigate whether this low percentage of real mature DC and a high percentage of macrophages in the alleged mature DC population is indeed NOD specific, we sorted mature $CD11c^{+}CD86^{+}$ DC from 7-day GM-CSF-stimulated cultures from NOD, NOR, C57BL, and BALB/c cultures and quantified the AP staining. As shown in Fig. 8*C*, NOD cells contained

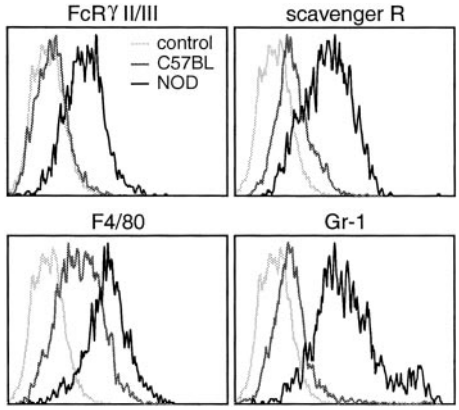

FIGURE 7. Elevated expression of monocyte/macrophage markers by mature NOD DC ($CD11c^{+}MHCII^{high}$) generated with GM-CSF. Cells were triple-labeled with CD11c, MHCII and a marker of interest. The light gray line indicates a histogram of the isotype control; the histogram indicated with the black line represents the marker expression by mature DC from NOD and the dark gray line of C57BL cells. Histograms are derived from a representative staining of at least six independent experiments per mouse strain.

Table II. *Intensity of monocyte/macrophage marker expression on CD11c$^+$MHCII^{high} DC from different mouse strains*

Marker	NOD (n = 5)[a]	NOR (n = 3)	C57BL (n = 4)	BALB/c (n = 1)
FcRγII/III	118 ± 53[b]	59 ± 6	38 ± 22	44
Scavenger R	162 ± 27	192 ± 6	11 ± 3	33
F4/80	148 ± 20	133 ± 8	28 ± 4	31
Gr-1	108 ± 26	70 ± 5	11 ± 4	7

[a] Number of samples derived from a representative staining
[b] Average mean fluorescence intensity. (± SEM).

significantly fewer cells with single-dot staining of mature DC than NOR, C57BL or BALB/c cells. However, both NOD and NOR contained more immature DC (weak cytoplasmic staining). Importantly, specifically CD11c$^+$CD86$^+$ DC from NOD cultures contained significantly more cells with strong cytoplasmic (macrophage-like) AP staining than any other strain tested.

Aberrant myeloid DC development from NOD BM precursors stimulated with Flt3-L

It might be argued that the aberrant response we observed mirrors the defect of NOD BM cells to respond to GM-CSF, rather than an inherent difficulty to develop into normal DC in culture. Flt3-L stimulates the development of both myeloid and plasmacytoid DC from mouse BM. Therefore, we cultured NOD, C57BL, and BALB/c BM with Flt3-L according to the method of Gilliet et al. (21)

Similar to our findings for GM-CSF-stimulated cultures, the yield at day 10 of the Flt3-L-stimulated cultures was lower from NOD precursors than from controls. In two independent experiments, the total cell yield in NOD cultures was 56 and 43% of

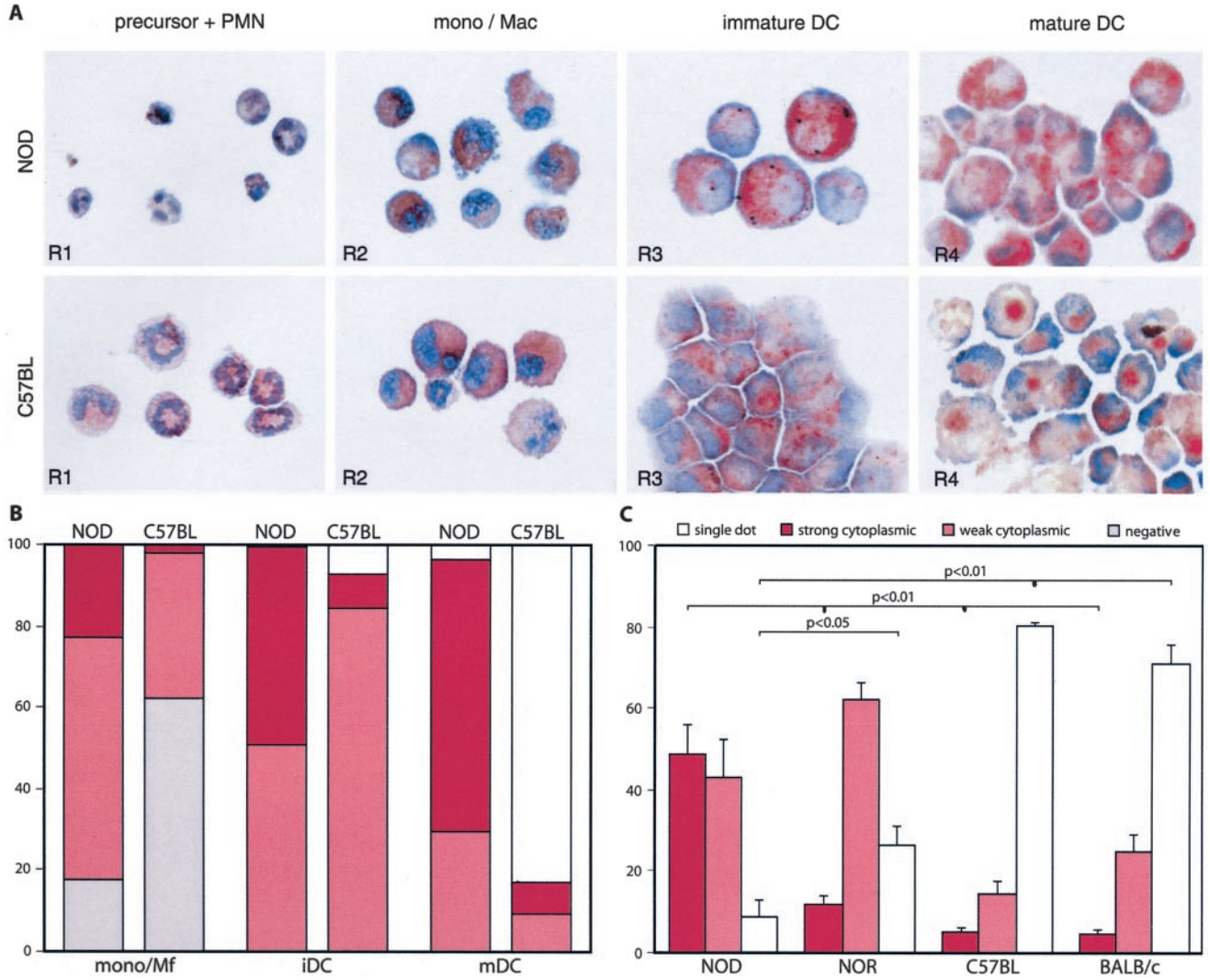

FIGURE 8. NOD mature DC have strongly increased acid phosphatase activity. *A*, Cytocentrifuge preparations of sorted cells were stained for AP and then counterstained with hematoxylin. PMN, Polymorphonuclear cells; mono, monocytes; Mac, macrophages. Magnification, ×285. *B*, Distribution of cells with different staining-pattern (see *Materials and Methods*) in three indicated sorted populations from the NOD and C57BL cultures. Populations with a phenotype of immature and mature DC from NOD cultures contained a high number of cells with a strong AP activity (red area of the bar). Mf, macrophages; iDC, immature DC; mDC, mature DC. *C*, Composition of the separated mature DC population (CD11c$^+$CD86$^+$) from four different mouse strains (NOD, NOR, C57BL, and BALB/c) with respect to the three cell types defined by the AP pattern (strong cytoplasmic staining, macrophages; weak cytoplasmic staining, immature DC; single-dot pattern, mature DC). NOD and NOR cells contained more immature DC and fewer mature DC as judged by their AP activity. High frequencies of cells with a high macrophage-like AP activity were found only in the NOD CD11c$^+$CD86$^+$ population. Data for *A* and *B* are representative of two independent experiments; *C* shows an average value ± SEM of four independent sortings for each mouse strain.

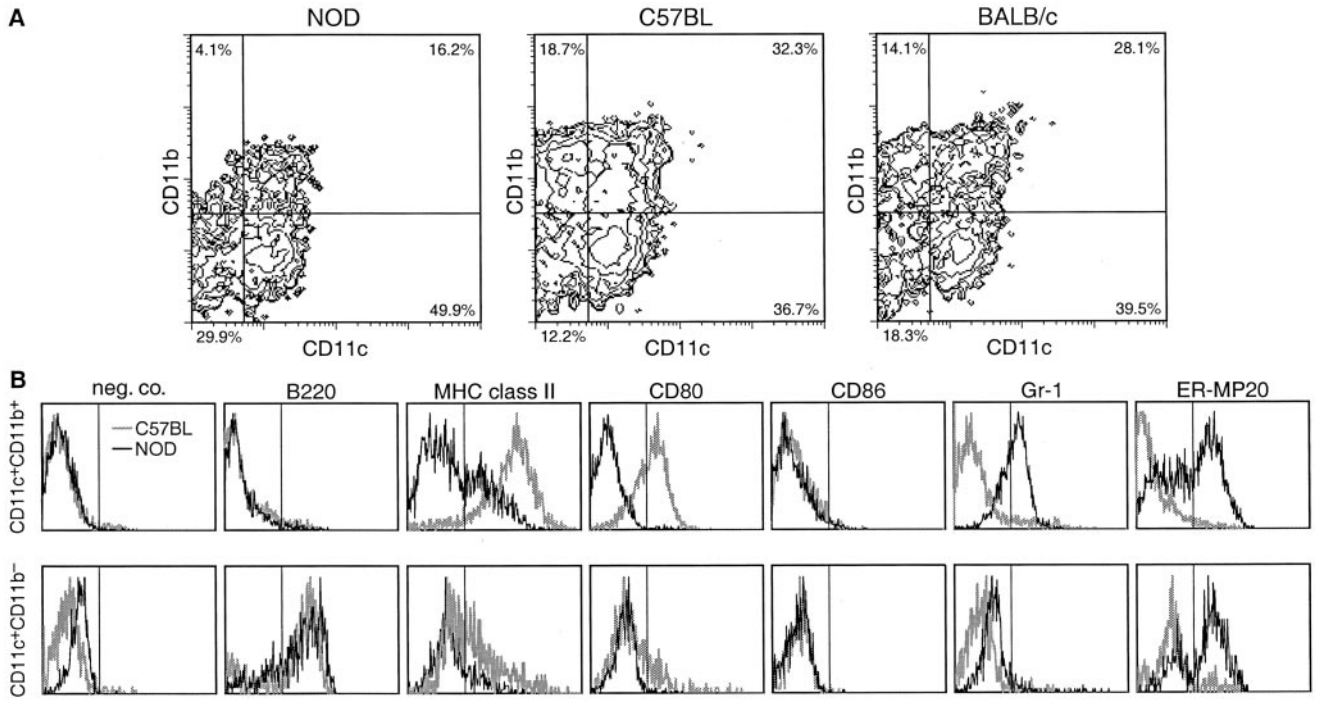

FIGURE 9. Aberrant generation of NOD myeloid DC in Flt3-L-stimulated cultures. BM cells were stimulated as reported by Gilliet et al. (21). *A*, Representative dot plots of CD11c/CD11b staining indicate the development of different DC subsets under the influence of Flt3-L. Numbers express the frequency of plasmacytoid ($CD11c^+CD11b^-$) and myeloid ($CD11c^+CD11b^+$) DC in NOD and control (C57BL and BALB/c) cultures. *B*, Histograms show the phenotype of plasmacytoid and myeloid DC in NOD vs control. Vertical line, Upper threshold of the negative control; black line, marker expression of gated plasmacytoid or myeloid DC in NOD culture; gray line, marker expression of the corresponding population in control culture. NOD myeloid DC displayed a similar deviant phenotype as observed in GM-CSF-stimulated culture. Plasmacytoid DC obtained from NOD and C57BL BM precursors had comparable phenotypes. Similar data were obtained in two independent experiments.

the C57BL and 67% of the BALB/c cultures. When analyzed by flow cytometry, the proportion and the phenotype of plasmacytoid $CD11c^+CD11b^-$ cells did not differ significantly among the strains (Fig. 9). In contrast, the NOD cultures contained particularly fewer myeloid $CD11c^+CD11b^+$ DC (16.2% in NOD vs 32.3 and 28.1% in C57BL and BALB/c, respectively) (Fig. 9*A*). Additionally, the phenotype of myeloid DC in NOD Flt3-L-stimulated cultures exhibited phenotypic abnormalities (Fig. 9*B*) similar to those in GM-CSF-stimulated cultures. NOD myeloid DC failed to reach a mature phenotype, unlike the control, in that they were primarily MHC class II^{low}, $CD80^-$, and $Gr\text{-}1^+$.

Therefore, the deviated development of myeloid DC in both Flt3-L and GM-CSF-stimulated cultures suggests an inherent abnormality in myeloid differentiation, rather than an aberrant response to a specific cytokine.

Discussion

The results presented in our study demonstrate several anomalies in the generation of myeloid DC from BM precursors in vitro in NOD and NOR mice: 1) a low cell yield from BM precursors was specific for the NOD and not found in NOR mice; 2) both NOD and NOR showed an increased cell death of BM DC precursors, but this was more outspoken in the NOD mouse; 3) an abnormally increased frequency of macrophages was found in GM-CSF-stimulated NOD and NOR BM cultures.

In addition, $CD11c^+MHCII^{high}$ DC with an aberrant phenotype and macrophage-like AP activity were generated in NOD cultures. We conclude that myeloid precursors from NOD mice stimulated with GM-CSF predominantly mature into macrophage-like cells. Similar phenotypic abnormalities were evident in NOD myeloid DC generated with Flt3-L, indicating an intrinsic deficiency of the precursors of mice with an NOD background in developing into real myeloid DC. The fact that only part of the NOD-associated anomalies in the generation of DC were present in the NOR underscores the multigenic complexity of the DC differentiation problems in mice of the NOD background.

Our first finding of the significantly lower yield of DC from NOD BM precursors in vitro is in agreement with previous reports (13, 14, 17). Furthermore, we demonstrated that in NOD/Ltj mice a combined low proliferation and an increased apoptosis of GM-CSF-stimulated BM precursors lead to such observed low cell yield. Our data concur with recently reported findings of a lower proliferation and an increased apoptosis of DC precursors in the congenic NOD $H\text{-}2^k$ mouse (31). Together, these data idncate an intrinsic problem of proliferation and apoptosis of DC precursors under the control of NOD-specific genes independent of the $H\text{-}2^{g7}$ MHC region. NOR mice that share several gene segments with the NOD mouse (including the $H\text{-}2^{g7}$ MHC region) (32) also shared part of the high apoptosis in the BM culture with NOD but had no problems in generating a normal cell yield.

Follow-up of DC maturation in NOD and NOR cultures revealed additional anomalies: BM-derived cells ineffectively differentiated into $CD11c^+MHCII^{high}$ prototypic DC and a significant proportion of $CD11c^+$ cells did not express MHC class II and costimulatory molecules. The latter cells were not immature DC incapable to bring MHC class II molecules to the cell surface, as evaluated by immunocytochemistry. Additionally, 24-h stimulation with LPS failed to induce maturation of these cells into typical mature DC. This, together with the morphology and phenotype of

the cells, supports the notion that the numerous $CD11c^+MHCII^-$ cells in NOD and NOR cultures were monocytes and macrophages and not DC. The codevelopment of macrophages during in vitro culture of BM precursors with GM-CSF has been reported previously in normal non-autoimmunity-prone mice (33). Likewise, we found $CD11c^+MHCII^-$ monocytes/macrophages in control cultures, but the frequency of these cells was very low in controls and noteworthy in NOD and NOR. Therefore, our data suggest that the decreased frequency of mature DC results from a deviated differentiation of myeloid precursors into macrophages (rather than DC) in BM cultures of mice with the NOD background.

A further abnormality we detected was that $CD11c^+MHCII^{high}$ $CD86^+$ DC from NOD cultures that did express costimulatory molecules and approached the phenotype of prototypic mature DC the best nevertheless had low T cell stimulation capacity, expressed increased levels of monocyte/macrophage markers, and had an abnormally high percentage of cells with a strong AP activity. These phenotypically mature DC therefore seemed more related to macrophages (hence we called these cells macrophage-like DC). Interestingly, DC from NOR mice shared properties with the NOD DC like a similar low maturation rate, a poor T cell stimulatory capacity, and some of the macrophage characteristics, such as the higher expression of scavenger R and F4/80. However, NOD cultures exclusively contained a high percentage of macrophage-like DC as judged by AP.

Our data once more indicate the complexity of the gene background determinants that establish various deviations in the development of DC from BM precursors in mice with the NOD background. According to Prochazka et al. (32), NOR mice possess C57BL/KsJ-derived genes on chromosomes 2, 4, 11, and 12. The remaining markers on 14 chromosomes, including the diabetogenic H-2g7 complex on chromosome 17, are of NOD origin. Interestingly, NOR mice, although diabetes resistant, share with NOD mice the T lymphocyte accumulation in peripheral organs and a depressed IL-1 secretion by peritoneal macrophages; unlike NOD, however, NOR mice have a robust suppressor T lymphocyte function.

The mechanisms that lead to the aberrant generation of DC in NOD cultures remain elusive. They might originate from intrinsic and genetically determined defects in BM precursors, or they might be induced by the numerous macrophages present and arising in the NOD cultures. Studies have been published that provide circumstantial support for each of the two possibilities (34–40).

With regard to the intrinsic defects in BM DC precursors, a deficient up-regulation of the GM-CSF receptor or an abnormal downstream signaling from the receptor are good candidates for the abnormal cell maturation and increased cell death in culture, particularly in view of the defective response of NOD myeloid precursors to GM-CSF and IL-5, which share the signaling chain of the receptor (34). However, we show that aberrant myeloid DC also develop when Flt3-L is used as the stimulator, signaling through a different receptor. Hence, we consider aberrations in a common signaling pathway more likely. Several studies thus far have shown abnormalities in NF-κB degradation in NOD splenocytes and in vitro-derived NOD APC (35, 36). It would be interesting to investigate whether similar defects can also be found in NOD and NOR DC precursors, thus at an earlier developmental stage.

Alternatively, the numerous macrophages present in the culture might inhibit a normal maturation of DC in the NOD and NOR cultures. Presence of macrophages in a culture is usually considered to be beneficial because they produce TNF-α and thereby support spontaneous maturation of DC (37). However, the abnormal cytokine profile that has been shown for NOD macrophages (38) might make them inhibitory instead of supportive for the growth and maturation of DC in culture. A potential inhibitory cytokine might be IL-6 as it has been shown that IL-6 shifts the differentiation of human monocytes from DC toward macrophages, whereas TNF-α favors maturation into DC (39, 40); however, in the supernatants collected from day 7 BM cultures, we could not detect appreciable levels of TNF-α, IL-6, or IL-1β, and when stimulated with LPS, C57BL and NOD BM-derived cells produced similar levels of these cytokines (data not shown). Therefore, it is less likely that an increased production of inhibitory cytokines in NOD cultures is the decisive factor in the deviated DC generation. However, macrophages in the GM-CSF-stimulated NOD and NOR cultures could still inhibit DC maturation by direct cell-cell interactions or by soluble factors produced at earlier stages in the culture.

An important difference between our current investigation and previous studies, in which an increased generation or enhanced stimulatory capacity of DC generated from NOD BM have been reported (15, 16), is that we did not use IL-4 in our culture system. A supportive effect of IL-4, added to GM-CSF, for the generation and maturation of NOD BM-derived DC was observed in several studies before (13, 14, 41, 42). Also, in our hands, NOD cultures showed an improved DC yield and stimulatory capacity in MLR when cultured with IL-4 added to GM-CSF (our unpublished observation). Normally, IL-4 is not added to GM-CSF-driven DC culture in mice because GM-CSF alone is considered sufficient for the optimal generation of DC from the mouse BM (20). However, unlike other mouse strains, BM precursors from NOD mice apparently critically depend on the presence of IL-4 for a normal and optimal development of DC. IL-4 inhibits macrophage outgrowth in the culture (43), but it may also function by increasing the sensitivity of the cells for DC maturation factors such as TNF-α (44). Whatever the mechanism may be, the beneficial effect of IL-4 in NOD cultures suggests a subtle intrinsic deficiency in NOD BM precursors for development into mature DC. This deficiency, magnified in vitro, might not be a decisive factor leading to obviously malfunctioning DC in vivo but probably contributes to the sensitivity of the DC system in NOD and NOR mice, which may be more prone to imbalance when challenged by external triggers in the NOD and protected by C57BL-inherited genes in the NOR mouse. In such a way, this subtle abnormality in DC differentiation might contribute to the autoimmune-prone genetics of NOD mice. However, because these anomalies are also largely present in NOR mice, they are certainly not sufficient to precipitate autoimmune diabetes on their own.

It is unclear whether the correlate of a macrophage-like DC that we find in NOD BM culture exists in vivo. GM-CSF-stimulated culture might be considered a system in which inflammation-related DC are generated; therefore, one could imagine that NOD myeloid precursors could encounter similar problems to undergo normal maturation during inflammation in vivo. The immature $CD11c^+MHCII^{low}$ DC produced under such circumstances would be the correlate of our macrophage-like DC in vitro. Findings from a recent study support this idea because NOD cells increasingly develop into $CD11c^+MHCII^{low}$ DC when reconstituting mixed bone marrow chimeric mice (31). Further studies on development and function of different DC subsets in the NOD mouse in steady state and inflammation would be necessary to confirm or reject this hypothesis.

In conclusion, in this study we describe an anomaly of myeloid BM precursors in NOD and NOR mice to develop preferentially into macrophages and abnormal macrophage-like DC when stimulated in vitro. Potentially, this defect contributes to the dysfunctional regulation of tolerance in NOD mice.

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