Signs of Immaturity of Splenic Dendritic Cells from the Autoimmune Prone Biobreeding Rat: Consequences for the In Vitro Expansion of Regulator and Effector T Cells[1]

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From the biobreeding-diabetic prone (BB-DP) rat, an animal model for endocrine autoimmunity, phenotype and function of splenic dendritic cells (DC) were studied. Furthermore, the suppressive effect of peritoneal macrophages (pMϕ) from the BB-DP rat in the MLR was investigated. Lower numbers of splenic DC were isolated from BB-DP rats than from control Wistar rats. In the preautoimmune phase, DC of the BB-DP rat had a lower surface MHC class II expression (and in preliminary data, a lower CD80 expression), ingested more bacteria, and had a lower stimulatory potency in the syngeneic (syn)MLR as compared with control DC. During disease development, the MHC class II expression further decreased, and a low stimulatory activity became evident in the allogeneic (allo)MLR. With regard to the expansion of suppressor/regulatory T cells, a lower percentage of RT6$^+$ T cells but higher percentages of CD45RC^{low} T cells were induced by BB-DP DC in synMLR, but not in alloMLR. An increase in the CD4/CD8 T cell ratio was observed in both the syn- and alloMLR due to a relative weak expansion of CD8$^+$ T cells with DC of the BB-DP rat. Resident pMϕ isolated from BB-DP or Wistar rats were equally effective in suppressing the DC-driven synMLR. In conclusion, splenic DC from the BB-DP rat have a lower accessory cell function already at young age, before the development of disease, and expanded different subsets of effector/suppressor T cells in vitro as compared with those from Wistar rats. The dysfunction of DC from BB-DP rats is likely to be caused by their relative immaturity as indicated by their low class II and costimulatory molecule expression and relatively high phagocytic activity. *The Journal of Immunology,* **1999, 162: 1795–1801.**

In animal models that spontaneously develop endocrine autoimmune disease (EAD),[3] such as the biobreeding-diabetic prone (BB-DP) rat and the nonobese diabetic (NOD) mouse, the thyroid gland and pancreas are infiltrated by MHC class II$^+$ dendritic cells (DC) in the preautoimmune phase (1–6). This initial accumulation of DC is followed by an enlargement of the draining lymph nodes and the production of autoantibodies by plasma cells located in these lymph nodes (1, 3). In patients with type 1 diabetes mellitus and autoimmune thyroid disease, DC also infiltrate the target glands (1, 7–9).

The local accumulation of DC early in the development of disease suggests a pivotal role of these cells in the pathogenesis of EAD. In patients with EAD, a defective maturation and function of DC has been reported: in diabetic patients, there is a loss of MHC class II$^+$ Langerhans cells from the epidermis at the onset of diabetes (10) and a reduction in the number of DC generated from monocytes, the precursor population in the peripheral blood (11). These monocyte-derived DC had a low stimulatory capability in autologous (auto)MLR (11), which was also described for unfractioned APC populations from the blood of diabetic patients (12–15). Furthermore, monocyte-derived DC from patients with Graves' disease and from diabetic patients showed a reduction in cellular interactions, i.e., the cells formed less homotypic clusters and clusters with T cells as compared with DC from healthy controls (11, 16).

A defective maturation and function of APC has also been described in the NOD mouse. APC precursors in the bone marrow showed a weakened maturational response to the growth factors IL-3 and CSF-1 (17), and splenic APC demonstrated a low stimulatory capacity in the syngeneic (syn)MLR (18, 19). Hence, these findings are—as in the human—indicative for a maturational defect of APC underlying the poor stimulatory capability. Indeed, immature DC have been recognized as relative poor T cell stimulators as compared with mature and activated DC (20, 21). Apart from intrinsic maturational defects, a reduced function of APC can also be caused by an increased production of suppressive factors from other monocyte-derived cells, i.e., macrophages (Mϕ). Mϕ from NOD mice and BB rats are able to produce high amounts of suppressive factors such as prostaglandins and nitric oxide (22–29).

Optimal APC function is required for appropriate stimulation of suppressor forces (12, 18). The reduced accessory cell function in auto/synMLR was regarded, both in the NOD mouse and in the patients, as a sign of a faulty capability to expand suppressor/regulatory T cells. The advantage of studying the BB rat is the presence of a well-defined suppressor/regulatory T cell population expressing the Ag RT6. These T cells play an important regulatory role in the pathogenesis of EAD in the BB-DP rat (30). BB-DP rats lack RT6$^+$ T cells and injections of these cells into BB-DP rats prevents EAD (31). BB-diabetic resistant (DR) rats do not develop

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[3] Abbreviations used in this paper: EAD, endocrine autoimmune disease; BB-DP, biobreeding-diabetic prone; NOD, nonobese diabetic; DC, dendritic cell(s); auto, autologous; syn, syngeneic; Mϕ, macrophage; DR, diabetic resistant; allo, allogeneic; pMϕ, peritoneal Mϕ; RPMI$^+$, RPMI 1640 with glutamax-1 and HEPES; FCSi, inactivated FCS; AP, acid phosphatase; PE, phycoerythrin; MFI, mean fluorescence intensity.

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diabetes and thyroid autoimmunity because of the presence of $RT6^+$ T cells, but depletion of these cells induces EAD (32). Thus, the BB rat is an excellent model to study putative functional defects of DC in relation to a defective suppressor/regulatory T cell expansion. We hypothesized that the loss of suppressor/regulatory T cells in BB-DP rats, which is already evident at young age, might be at least in part due to a defective function of DC before the development of EAD. Therefore, the function of splenic DC from BB-DP rats of different ages in allogeneic (allo)MLR (with Lewis T cells) and synMLR (with Wistar and BB-DP T cells) was studied. In particular, we were interested in BB-DP rats of ages 3–7 wk, i.e., before the DC infiltration into the thyroids and pancreas islets and before EAD development. We focussed on the in vitro expansion of $RT6^+$ T cells within a normal T cell population from Wistar and Lewis rats. Because $CD8^+$ cells and $CD45RC^{low}$ cells have also been associated with regulatory T cells in the BB rat (33, 34), CD4/CD8 ratios of the in vitro expanded T cells were calculated and CD45RC expression was examined. DC of the BB-DP rat proved to be less capable in generating $RT6^+$ and $CD8^+$ T cells, and this could be due to an immature phenotype. For this purpose, the uptake of dextran molecules and bacteria was studied (20, 21), together with the expression of MHC class I and II, costimulatory and adhesion molecules, the DC marker OX-62, and the DC/Mϕ markers, ED1 and ED7. Finally, we studied the ability of BB-DP Mϕ to reduce accessory cell function by adding resident peritoneal Mϕ (pMϕ) to synMLR driven by splenic DC of the Wistar rat.

Materials and Methods

Animals

Male and female BB-DP rats were bred in the Experimental Animal Center of the Erasmus University (Rotterdam, The Netherlands) and control Wistar rats were purchased from Harlan (Zeist, The Netherlands). All rats were kept under controlled light conditions (12/12 h light/dark cycle) throughout this study. A standard pelleted diet (0.35 mg iodine/kg; AM-II, Hope Farms BV, Woerden, The Netherlands) and tap water were provided ad libitum. Between 12 and 20 wk of age, 90% of our BB-DP rats developed α-colloid Abs detected in serum, and 70–80% of the rats became diabetic. BB-DP rats were daily tested for glucosuria (Gluketur test sticks; Boehringer Mannheim BV, Almere, The Netherlands). For all experiments, age matched BB and Wistar rats were used.

Cell preparations

Splenic DC were enriched according to the method of Knight et al. (35), with slight modifications. Briefly, spleens from BB-DP and Wistar rats were minced and digested for 1 h at 37°C in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) with 25 mM glutamax-1 and 25 mM HEPES (hereafter referred to as $RPMI^+$) containing: 125 U/ml collagenase (type III; Worthington Biochemical, Freehold, NJ) and 0.1 mg/ml DNase (Boehringer Mannheim BV). The remaining tissue was teased through a 105-μm filter, and the erythrocytes were removed by lysis. Finally, the separated cells were washed and cultured in $RPMI^+$ supplemented with 10% inactivated FCS (FCSi), penicillin (100 U/ml; Seromed, Biochrom, Berlin, Germany), and streptomycin (0.1 mg/ml; Seromed). After an overnight culture period in culture flasks (Costar Europe, Badhoevedorp, The Netherlands; 37°C, 5% CO_2 incubator), DC were isolated from the nonadherent cells by using a 14.5% (w/v) Nycodenz (Nycomed Pharma As, Oslo, Norway) density gradient (800 $\times$ *g* for 20 min). Low-density cells were collected from the interphase and washed. This cell fraction demonstrated a dendritic morphology, a strong MHC II expression (see *Results*), and a weak acid phosphatase (AP) activity in 70–95% of cells in both rat strains.

Resident pMϕ were harvested by lavage of the peritoneal cavity of BB-DP and Wistar rats with 10 ml ice-cold PBS, pH 7.4, containing 50 U/ml heparin. Cells were washed, and, when present, erythrocytes were lysed. The peritoneal fluid contained, in both rat strains, 50–63% $ED2^+$ and AP^+ cells as determined on cytospin preparations.

T cells from BB-DP, Wistar, and Lewis rats were enriched using a nylon wool column. In short, spleens were minced and teased through a 105-μm filter, and the erythrocytes were removed by lysis. Cells were washed and loaded onto a nylon wool column (3 g; Polyscience, Eppelheim, Germany) packed into a 60 ml plastic syringe. After 1 h in 5% CO_2 incubator, T cells (80–90% $CD5^+$ cells) were harvested by collecting the effluent.

MLR

For the MLR, splenic DC from BB-DP and Wistar rats were irradiated with 2,000 rad and added at various ratios to T cells (fixed number of 150,000 T cells/well) in flat-bottom 96-wells plates (Nunc, Roskilde, Denmark). Subsequently, these MLR were cultured for 3 or 5 days in RPMI 1640 containing 50 mM HEPES buffer (Life Technologies), 10% FCSi, 110 μg/ml sodium pyruvate (Merck, Munich, Germany), 0.5% (v/v) β-mercapto-ethanol (Merck), and antibiotics. To determine the effect of pMϕ on accessory cell function, splenic Wistar DC were mixed with Wistar T cells (150,000 T cells/well) at a ratio of 1:20, and pMϕ of either BB-DP or Wistar rats were added at a DC:Mϕ ratio of 1:50, 1:10, and 1:2. As a control, accessory cell function of the pMϕ alone were determined. Next, cells were cultured for 5 days.

In the MLR, T cell proliferation was measured via tritiated thymidine ($[^3H]$TdR) incorporation (0.5 μCi/well during the last 16 h of total culture period). Finally, cells were harvested on filter papers, and radioactivity was counted in a liquid scintillation analyzer (LKB Betaplate, Wallac, Turun, Finland). For phenotyping (see below), T cells were collected and washed in PBS containing 0.5% BSA (Sigma, Axel, The Netherlands) and 20 mM sodium azide (Sigma).

Abs

The following mAbs were used: anti-MHC class I (1:400, MRC OX-18; Serotec, Oxford, England), anti-MHC class II conjugated to phycoerythrin (PE) (1:400; MRC OX-6, Serotec), anti-B7-1 (1:500, CD80; Research Diagnostics, Flanders, NJ), anti-B7-2 (1:500, CD86; Research Diagnostics), anti-rat DC-PE (undiluted, MRC OX-62; Serotec) anti-LFA-1α (1:10, CD11a; Serotec), anti-ICAM-1 (1:50, CD54; Serotec), anti-very late Ag-4 (VLA-4) (1:10, CD49b; Serotec), ED1 (1:400, monocytes, Mϕ), ED7 (1:10, monocytes, Mϕ; both ED Abs were a kind gift from Prof. Dr. C. D. Dijkstra, Department of Cell Biology, Free University, Amsterdam, The Netherlands), anti-CD5 (1:100, MRC OX-19; Serotec) anti-CD4 conjugated to biotin (1:4, Domain 1; Serotec), anti-CD8-PE (1:4, MRC OX-8; Serotec), anti-CD45RC (1:100, MRC OX-22; Serotec), anti RT6.1-FITC (1:10, 3G2, for staining Lewis T cells), and anti-RT6.2-Biotin (1:10, GY 1/12, for staining Wistar T cells; both anti-RT6 Abs were a kind gift from Dr. H. Groen, Department of Histology and Cell Biology, University of Groningen, Groningen, The Netherlands).

Cell staining

Cells were added in round-bottom 96-wells plates (Nunc) at a concentration of $\sim 10^5$ cells/well and washed twice in PBS/0.5% BSA/20 mM sodium azide. Pelleted cells were resuspended in 20 μl solution with labeled primary Abs, incubated for 10 min., and followed by two washing steps. Using unconjugated Abs, a second step was incorporated with rabbit anti-mouse-FITC Abs (Dako, Glustrup, Denmark) with 1% normal rat serum. For the visualizing of biotin-conjugated Abs, streptavidin-tricolor (Caltac Laboratories, San Francisco, CA) was used. For cell analysis, 10,000 events were recorded with a FACS (FACScan, Becton Dickinson, Sunnyvale, CA). Dead cells, recognized by their uptake of propidium iodide and their specific forward- and side-scatter pattern, were excluded from analysis. For determination of background staining, cells were incubated with either labeled irrelevant Abs or with secondary Abs or with streptavidin-tricolor alone.

Uptake of FITC-dextran

The uptake of FITC-dextran (Sigma) by DC was performed according to the method by Sallusto et al. (20). In short, DC were added to round-bottom 96-wells plates (Nunc) at a concentration of 10^5 cells/well in $RPMI^+$, 10% FCSi, and antibiotics. FITC-dextran was added at a final concentration of 1 mg/ml for 0, 15, 30, and 60 min. After incubation, the cells were put on ice and washed thrice. Finally, the cells were stained with OX-6-PE (Serotec) and analyzed on a FACScan.

Phagocytosis of FITC-labeled Escherichia coli

For studying the uptake of *E. coli* bacteria by DC, a phagotest (Becton Dickinson) was used according to the instructions of the manufacturer with some minor modification. Briefly, 20 μl precooled *E. coli* bacteria were added to suspension with DC (10^6 cells/ml in $RPMI^+$, 10% FCSi, and antibiotics). The controls remained on ice and the phagocytosis assay was incubated for 30′ and 60′ at 37°C in an incubator under rotation (4 rpm). Cells were put on ice, washed, and 100 μl quenching solution was added.

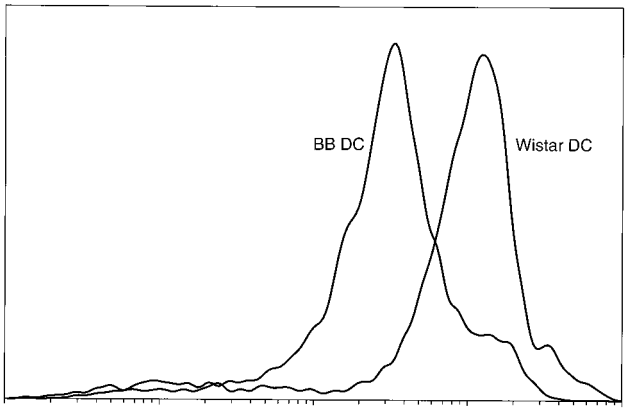

FIGURE 1. A histogram of MHC class II expression on splenic DC from BB-DP and Wistar rats (age: 11 wk).

Cells were washed thrice and stained with OX-6-PE (Serotec) and analyzed on a FACScan.

Statistical analysis

The results are presented as means ± SEM, and statistical analysis of the data was performed with the Mann-Whitney *U* test.

Results

Number and phenotype of isolated DC

The percentages of isolated splenic DC from BB-DP rats were significantly lower than from Wistar rats (1.0 ± 0.7% vs 1.4 ± 0.6%, $p = 0.003$, $n = 6$, age 3–20 wk). The purity of DC, relevant for performing further functional studies (see below), was measured by dendritic morphology and MHC class II expression, and did not differ for both rat strains (range 70–95%, data not shown).

In the preautoimmune phase, DC from BB-DP rats (age 3–7 wk) demonstrated a lower surface MHC class II expression than those from Wistar rats (mean fluorescence intensity (MFI) for Wistar and BB-DP rats, respectively, was 1586 ± 510 and 1084 ± 386, $p = 0.02$, $n = 23$). During development and expression of disease (age 8–20 wk), surface MHC class II expression further decreased on BB-DP DC (MFI for Wistar and BB-DP rats, respectively, was 1452 ± 642 and 533 ± 124, $p = 0.006$, $n = 19$; see also Fig. 1). DC from both rat strains had a weak AP activity located in a spot near the nucleus and did not differ in the percentage of positive cells and expression of the following surface markers: MHC class I molecules (MFI for Wistar and BB-DP rats, respectively, was 214 ± 61 and 231 ± 57, $n = 13$); adhesion molecules LFA-1 (73 ± 10 and 77 ± 13, $n = 3$), ICAM-1 (116 ± 19 and 114 ± 15, $n = 4$), and VLA-4 (65 ± 6 and 61 ± 11, $n = 3$); DC/Mϕ markers ED1 (57 ± 14 vs 55 ± 17, $n = 3$) and ED7 (80 ± 9 vs 72 ± 9, $n = 3$). DC from both rat strains were negative for CD4 and CD8. The rat DC marker OX-62 only showed a weak expression in our hands on both Wistar DC and BB-DP DC (data not shown).

CD80 and CD86 expression was also determined, but in a limited series of experiments. DC from BB-DP rats (age 3–7 wk) had a lower CD80 expression than those from Wistar rats (MFI for Wistar and BB-DP rats, respectively, was 192 ± 57 and 98 ± 56, $n = 3$). The expression of CD86 did not differ for both rat strain DC (214 ± 76 and 224 ± 82, $n = 3$).

Uptake of dextran and E. coli *bacteria*

Incubation with FITC-dextran resulted in an increased uptake in time by the DC and this tended to be higher for the BB-DP rat (age 3–20 wk) after 60 min. (MFI for Wistar and BB-DP rats, respectively, was 67 ± 9 and 81 ± 14, $n = 6$).

Table I. *Percentage of MHC class II$^+$ and FITC-E. coli$^+$ splenic DC from BB-DP and Wistar rats ($n = 4–6$) after incubation with E. coli bacteria.*

DC	Time (min) 0	30	60
Wistar	0.9 ± 0.3	3.7 ± 2.1	4.3 ± 1.5[a]
BB	1.7 ± 1.3	4.8 ± 2.3	12.2 ± 4.6

[a] $p = 0.03$, Wistar vs BB-DP.

During incubation with FITC-*E. coli* bacteria, DC showed an increased phagocytosis and after 60 min. significantly more bacteria were taken up by DC from the BB-DP rat (age 3–20 wk) as compared with the Wistar rat (Table I). DC from BB-DP rats of different ages (3–7 wk and 8–20 wk) did not differ in the uptake of the bacteria (data not shown). In contrast with DC, >85% of Wistar pMϕ, which are professional phagocytes, were loaded with FITC-*E. coli* bacteria after 60 min (data not shown).

Proliferation of T cells

After 3 days in syn- and alloMLR, no differences in the proliferation of T cells were observed with DC from either BB-DP or Wistar rats (data not shown). After 5 days, optimal proliferation was observed, and in the synMLR with Wistar T cells a significantly lower uptake of [^{3}H]TdR by the T cells (low proliferation) was found with DC from BB-DP rats compared with Wistar rats for both age groups of 3–7 and 8–20 wk (Fig. 2). Similar results were obtained in the synMLR with T cells from the BB-DP rat, although the overall counts were much lower due to the reduced proliferative capacity of these T cells (Fig. 2). Here, the number of experiments were too low ($n = 2$) to perform statistical analysis.

In the alloMLR, no differences in accessory cell function were observed between DC from BB-DP and Wistar rats in the age

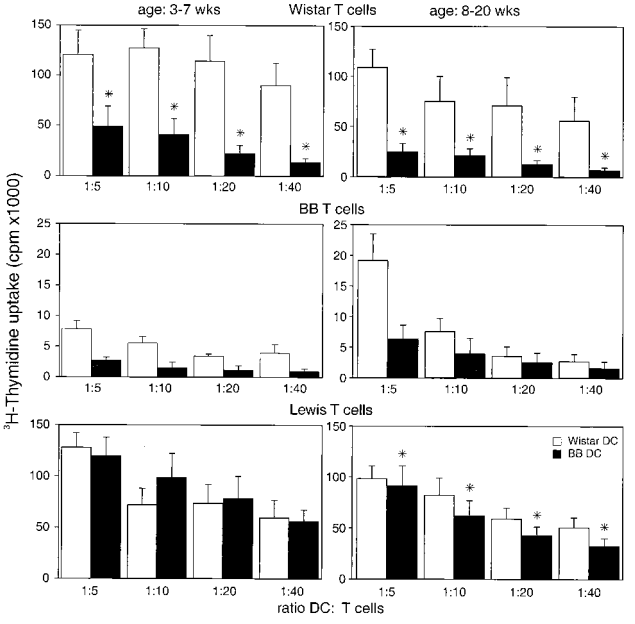

FIGURE 2. [^{3}H]TdR uptake by T cells after 5 days of coculture with splenic DC from BB-DP (■) and Wistar rats (□) (MLR with Wistar T cells: $n = 6–7$; *, $p < 0.03$ compared with Wistar for all DC: T cell ratios used and for all age groups; MLR with BB T cells: $n = 2$; MLR with Lewis T cells: $n = 9–10$; *, $p < 0.03$ compared with Wistar for all DC: T cell ratios in age group 8–20 wk).

Table II. *Percentages of RT6$^+$ and CD45RC$^+$ T cells and CD4/CD8 ratio after 5 days of coculture with splenic DC from BB-DP and Wistar rats (n = 6–13)*

	Percent RT6$^+$		Percent CD45RC$^+$		CD4/CD8 Ratio	
T cells	Wistar	Lewis	Wistar	Lewis	Wistar	Lewis
Wistar DC	57.0 ± 8.4[a]	48.9 ± 2.9	47.0 ± 5.2[a]	16.5 ± 2.6	1.4 ± 0.2[a]	1.3 ± 0.1[a]
BB DC	43.6 ± 7.4	43.0 ± 3.2	33.2 ± 4.7	15.2 ± 2.1	3.5 ± 0.4	2.0 ± 0.3

[a] $p < 0.02$, Wistar vs BB-DP.

group of 3–7 wk (Fig. 2). In contrast, BB-DP DC induced significantly less proliferation of Lewis T cells than Wistar DC in the age group of 8–20 wk (Fig. 2). With DC isolated from lymph nodes, similar results in the alloMLR for the different strains were observed (data not shown).

Expansion of T cell subsets in DC-driven MLR

No differences in the expansion of T cell subsets were observed on day 3 in the syn- and alloMLR (data not shown). On day 5 of these MLR, a significant increase in the CD4/CD8 ratio with DC from the BB-DP rat was observed (Table II). In the synMLR with T cells from the BB-DP rat, similar observations were made: the CD4/CD8 ratio with DC from Wistar and BB-DP rat was 0.7 ± 0.2 and 2.1 ± 0.5, respectively (n = 2). In synMLR, significant lower percentages of CD45RC$^+$ and RT6$^+$ T cells were induced by BB-DP DC as compared with Wistar DC. This was not observed in the alloMLR using Lewis T cells (Table II). An example of CD4, CD8, and RT6 expression on Wistar T cells cultured with DC from Wistar and BB-DP rats is given in Fig. 3.

Effect of peritoneal macrophages in the synMLR

Different concentrations of pMϕ were cocultured with DC and T cells from the Wistar rat to study their effect in the synMLR. Culture of DC together with T cells (DC:T cell ratio of 1:20) gave >40,000 counts, whereas culture with pMϕ alone with T cells resulted in a much lower T cell proliferation (Fig. 4). The addition of pMϕ in the synMLR at a DC:Mϕ ratio of 1:50 and 1:10, resulted in a similar inhibition of T cell proliferation for both rat strains. No significant inhibitory effect on the DC-driven MLR was found using lower numbers of pMϕ (DC:Mϕ ratio of 1:2; Fig. 4).

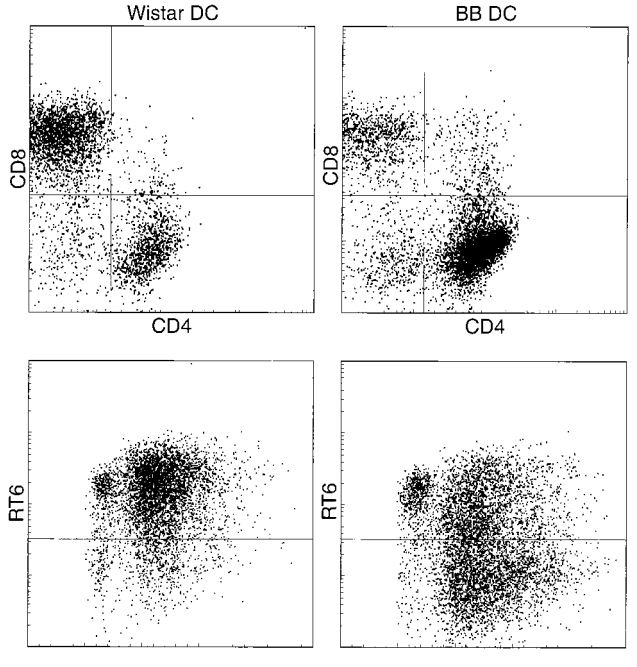

FIGURE 3. Expression of CD4, CD8 (in a dotplot), and RT6 (fluorescence combined with forward scatter) on Wistar T cells after 5 days of coculture with splenic DC from BB-DP or Wistar rats in the synMLR.

Discussion

DC form a heterogeneous population of bone marrow-derived cells capable of providing immunizing and tolerizing signals for T cells. Lymphoid DC, first described in the thymus, are able to restrict T cell responses (36, 37). Myeloid DC induce T cell responses, both of effector (38) as well as of regulator/suppressor forces (39–41), and can be generated from CD14$^+$ monocytes (42). Monocyte-derived myeloid DC are immature when residing in peripheral tissues, but fully mature during traffic from the periphery to the lymphoid organs. Signals for maturation can be delivered by monocytes (monokines), lymphocytes (cytokines, CD40 ligation), and bacteria (LPS). Immature myeloid DC are characterized by a relative high capability to take up foreign (antigenic) material, whereas mature forms are better equipped to stimulate T cells (21).

This study demonstrates that splenic DC from the BB-DP rat show signs of immaturity as indicated by the following. First, a low expression of surface MHC class II molecules and also of CD80 together with an enhanced uptake of bacteria is shown. These abnormalities occurred already in the preautoimmune period (3–7 wk of age) and, with respect to MHC class II expression, further decreased in the autoimmune period (8–20 wk of age). Although not analyzed in this study, determination of intracellular class II expression would help to substantiate immaturity by differences in the distribution of MHC class II molecules (20, 21).

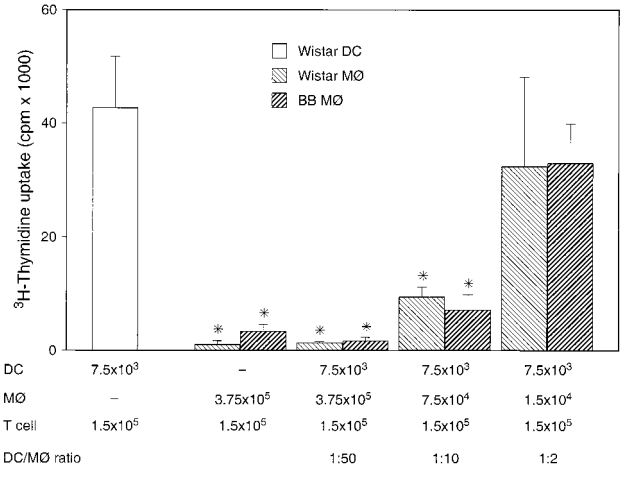

FIGURE 4. [^{3}H]TdR uptake by Wistar T cells after 5 days of coculture with splenic Wistar DC and/or pMϕ from BB-DP and Wistar rats (n = 7–11; *, $p < 0.01$ for differences in T cell proliferation by Wistar DC as compared with pMϕ alone or DC combined with pMϕ at a ratio of 1:50 and 1:10 for both rat strains).

Second, a lower T cell stimulatory capacity in the synMLR for all periods studied, and also in the alloMLR in the autoimmune period is shown.

In addition, BB-DP splenic DC were also less in number upon isolation, which may be taken as a sign for a reduced generation/maturation of DC from their precursors. Further studies with monocyte-derived and bone marrow-derived DC and the effect of maturation stimuli like LPS are planned, as these would best answer the issues regarding defective DC maturation and function in the BB-DP rat.

The described DC dysfunction before the onset of autoimmunity, i.e., before the accumulation of DC in the thyroids and pancreas islets of the BB-DP rat, is suggestive for an innate defect, which may form the basis for the development of EAD and aggravates during aging/disease. Interestingly, similar impaired function of DC has recently been described in the preclinical phase of human diabetes (43).

How do our findings relate to other studies on DC and APC function in the BB-DP rat? In a similar study, but using another subline of BB-DP rats, Tafuri et al. (44) reported the same level of MHC class II expression and accessory cell activity of splenic DC from BB-DP and Wistar rats. However, these authors only studied young rats (age 3–7 wk) and tested their DC in alloMLR but not in synMLR. Also, we could not demonstrate DC defects in alloMLR at such young age. In other studies, using nonpurified APC populations (45, 46), the data are sometimes confusing, and comparison with our data is difficult. In contrast with our findings, Bellgrau et al. (45) reported, using unfractioned lymph node cells, a normal synMLR, although these APC induced a lower response in the alloMLR. It is not clear from their report whether experiments were performed with either young or old rats. Also Elder et al. (46) described that alloMLR driven by unseparated BB-DP spleen cells was lower, as compared with those driven by Wistar rat APC. But, higher T cell stimulations with BB-DP DC could be obtained depending on the conditions of the experiment and the concentration of the stimulator cells (46). In addition, it must be noted that the unfractioned APC populations used in the above-cited studies may contain Mϕ, which are likely to affect the proliferation of the responder T cell population (see present data and Refs. 22, 23, and 28).

Both in diabetic patients and in NOD mice, the failure of DC to sufficiently stimulate T cells in auto/synMLR has been considered as reflecting a decreased capability of these cells to expand regulatory/suppressor T cells (12, 18). Indeed, in the present study, the reduced accessory cell function of the immature splenic DC from the BB-DP rat resulted in the following conditions. First, a low generation of $RT6^+$ T cells in synMLR is found. The $RT6^+$ population has been reported to contain cells that protect BB rats against the development of EAD (31, 47). RT6 is a glycosyl-phosphatidylinositol-linked surface molecule present on ~70% of mature peripheral T cells (48, 49). The exact mechanism of protection is not clear but the RT6 molecule generates an activation signal, which may influence T cell function (50). Second, an increased CD4/CD8 ratio, due to a primary expansion of $CD4^+$ T cells, not only in synMLR (when BB-DP T cells or Wistar T cells were used as responder cells), but also in allogeneic responses (Lewis T cells), is found.

Taken together our data showing a low in vitro expansion of $RT6^+$ T cells and of $CD8^+$ T cells (in relation to $CD4^+$ T cells) by immature BB-DP splenic DC supports our hypothesis that DC with a defective T cell stimulatory function, which can also be found in human type 1 diabetes, have an inability to generate sufficient suppressor forces (see *Introduction*). However, some words of caution are necessary.

First, in our experiments with BB-DP DC, we have not made a comparison with splenic DC and/or T cells of BB-DR rats to link the maturation defect of DC to EAD development and to avoid minor histocompatability differences in synMLR. A DR variant of our BB-DP Rotterdam strain does not exist, but in a few preliminary experiments we compared accessory cell function of splenic DC of BB-DP and BB-DR rats of the Worcester strain. Interestingly, DC of both Worcester strains did not show the defects as observed with DC of the Rotterdam strain. In this respect, it is of note that our BB-DP strain has a relatively high prevalence of thyroiditis (51, 52). We did find other signs of immaturity of DC from the Worcester sublines: a lower homotypic cluster capability as compared with the Wistar rat, and the BB-DP rat was more affected than the BB-DR rat. At present, these data are subject for further study.

Second, suppressor activity has not only been recognized in the $RT6^+$ and $CD8^+$ T cell population, but also in a population of $CD45RC^{low}$ T cells (33, 47), and, within the $CD4^+$ T cells, these cells have been recognized as Th2-like cells, producing IL-4 (33). In our study, DC from the BB-DP rat induced not less, but more of these "suppressor" $CD45RC^{low}$ T cells in vitro as compared with the Wistar rat. Furthermore, the $CD45RC^{high}/CD45RC^{low}$ ratios of both $CD4^+$ and $CD8^+$ T cell subsets did not differ in the MLR for both rat strains using either Wistar or Lewis T cells (data not shown). On the other hand, not only $CD45RC^{low}$ T cells may act as suppressor/regulatory cells; also $CD4^+$ $CD45RC^{high}$ Th1-like cells can act as regulator cells suppressing graft rejection in the Lewis rat (53). Hence, protective cell populations other than $CD8^+$, $RT6^+$, or $CD45RC^{low}$ T cells are likely present in the BB-DP rat. In addition, transplantation of thymic tissue from DR rats into BB-DP rats prevented the development of diabetes without restoration of the $RT6^+$ T cell population (54). Nagata et al. (55) reported the prevention of diabetes in BB-DP rats with a $CD4^+CD8^-$ and $RT6^-$ T cell line, but it is not clear whether this cell line may obtain the RT6 phenotype after transfer. The present studied showed that DC from the BB-DP rat expanded different subsets of effector and suppressor/regulatory T cell populations in vitro as compared with Wistar control rats. The question remains whether BB-DP DC are indeed less capable of expanding T cells that suppress EAD in vivo. This question may be solved by studying the effect of in vitro expanded T cells (in synMLR) on the development of EAD after transfer in BB-DP rats.

The mechanisms of the defective maturation of splenic DC from the BB-DP rat could hypothetically reside on the level of the precursor in the bone marrow, as observed for NOD APC (17), or on other levels, such as a lack of maturation signals. The latter is illustrated by the transition from blood monocyte to full mature DC induced by lymphocyte-derived cytokines and lymphocytic contacts (CD40 ligation). Already at young age, the BB-DP rat becomes severely lymphopenic (46, 56, 57), resulting in a strong reduction in the number of especially $CD8^+$, $CD45RC^+$, and $RT6^+$ T cells (58–60). This reduction is at least partly due to genetically encoded defects in intrathymic T cell maturation (60–62). Hence, the T lymphopenia of the BB-DP rat could be primary and induce a faulty cross-talk between lymphocytes and DC leading to a poor development and maturation of both T cells and DC. The observation that DC from BB-DP rats (age 3–7 wk) stimulated the alloMLR equivalently to Wistar DC (see Fig. 2), suggests that the maturation and function of BB-DP DC can be restored with "normal" T cells. Indeed, preliminary data showed a restoration of homotypic cluster formation of BB-DP DC after incubation with factors produced by Con A-stimulated T cells. Whether MHC class II and CD80 expression is also restored is now under investigation. We favor the idea that the defective cross-talk between DC and T

cells of the BB-DP rat resides on both the intra- and extrathymic level. Extrathymic defects in T cell maturation in the BB-DP rat have been suggested before. With respect to the defective intrathymic T cell maturation in the BB-DP rat, a role for thymic APC has also been recognized: introduction of a bone marrow-derived thymic APC fraction from DR donors into the thymus of the BB-DP rat restored the T cell proliferative function and prevented disease. However, no restoration of the T lymphopenia and RT6 expression was achieved (54).

Mϕ-derived factors may also play a role in the defective DC maturation in BB-DP rats. One of the major maturation-inducing cytokines for DC, TNF-α (21, 63, 64), is produced in much lower quantities by pMϕ of BB-DP rats than by BB-DR rats (65). Treatment with or induction of TNF-α with CFA decreased the incidence of diabetes in the BB-DP rat (66, 67). One could speculate whether such treatment would induce a full maturation of BB-DP DC and subsequently up-regulate their accessory cell function in MLR. However, preliminary data showed that culture of splenic BB-DP DC in the presence of a single dose of rat or human TNF-α and/or combined with another maturation-inducing cytokine human granulocyte-Mϕ CSF, had no effect on their accessory cell function in the MLR (data not shown).

Defective function of BB-DP splenic DC could also be due to an excessive production of suppressive factors by BB-DP Mϕ, but pMϕ of BB-DP and Wistar rats had similar inhibitory activities on the synMLR driven by splenic DC of Wistar rats. These data are in contrast to some previous reports demonstrating a stronger suppression of APC by Mϕ of the BB-DP rat and an enhanced production of suppressive factors (22, 23, 27, 28). However, other data show the opposite: the addition of BB-DP Mϕ enhanced the stimulatory activity of DC (44). These conflicting data may be due to the usage of different populations of Mϕ (either isolated from the spleen or the peritoneum) and differences in the activation state of these Mϕ (e.g., exudate vs resident pMϕ, the latter being strongly affected by the isolation procedure). In our study, cells were harvested from the peritoneum without further purification steps, like adherence or gradient centrifugation (22, 23, 27, 28), ensuring a minimal degree of activation. If indeed BB-DP Mϕ have a stronger suppressive effect (22, 23, 27, 28), this would further limit the already depressed BB-DP DC activity.

In conclusion, the present study gives evidence that splenic DC from the BB-DP rat have an immature phenotype and an impaired function before the development of endocrine autoimmunity. Furthermore, these defective DC induce a different expansion of T cell subsets in vitro and the question remains whether these APC defects also lead to a faulty in vivo induction of tolerance/suppression. The putative mechanisms underlying this altered maturation (defective bone marrow factors, T cell defects) remain to be elucidated.

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