Plasmacytoid dendritic cells are proportionally expanded at diagnosis of Type 1 diabetes and enhance islet autoantigen presentation to T cells through immune complex capture

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ABSTRACT

Objective. Immune-mediated destruction of β-cells resulting in type 1 diabetes involves activation of pro-inflammatory, islet autoreactive T-cells, a process under the control of dendritic cells of the innate immune system. We tested the hypothesis that type 1 diabetes development is associated with disturbance of blood dendritic cell subsets that could enhance islet-specific autoimmunity.

Research Design and Methods. We examined blood dendritic cells (plasmacytoid, myeloid) in 40 patients with recent-onset diabetes (median duration 28 days) and matched control subjects. We also examined the relative ability of different dendritic cell subsets to process and present soluble or immune complexed islet cell autoantigen (the islet tyrosine phosphatase IA-2), to responder CD4 T-cells.

Results. The balance of blood dendritic cells was profoundly disturbed at diabetes diagnosis, with significant elevated proportion of plasmacytoid and reduction of myeloid cells compared with control subjects. Dendritic cell subset distribution was normal in long-standing disease and in patients with type 2 diabetes. Both dendritic cell subsets processed and presented soluble IA-2 to CD4 T-cells after short-term culture, but only plasmacytoid dendritic cells enhanced (by as much as 100%) autoantigen presentation in the presence of IA-2 autoantibody-positive patient serum.

Conclusions. The plasmacytoid subset of dendritic cells is over-represented in the blood close to diabetes onset and shows a distinctive ability to capture islet autoantigenic immune complexes and enhance autoantigen-driven CD4 T-cell activation. This suggests a synergistic pro-inflammatory role for plasmacytoid dendritic cells and islet cell autoantibodies in type 1 diabetes.
Type 1 diabetes is an autoimmune disease resulting from T cell-mediated destruction of insulin-producing β-cells (1-3). Whilst the precise aetio-pathogenesis of the disease is unknown, it is apparent that β-cell damage involves the generation of activated, pro-inflammatory, islet-autoreactive, effector CD4 and CD8 T cells (3; 4). The priming, differentiation and expansion of effector T cells is largely under the control of a heterogeneous group of immune cells that go under the collective term of dendritic cells, because of their distinctive morphology (5). Dendritic cells (DCs) have numerous specialized forms present in peripheral tissues, lymph nodes and the blood and, collectively, these cells are responsible for the sensing and ingestion of pathogens and activation of T cells of relevant specificity. Since activated DCs are a requirement for priming of naïve T cells, it is likely that a similar process pertains during the development of islet autoreactivity, although the activating stimuli and islet autoantigens involved remain obscure. It is also likely that once this process is initiated, DC presentation of islet autoantigens remains a feature of the disease, as spreading of the autoimmune response to additional autoantigens and epitopes develops (6).

Given the pivotal role of DCs in the activation of naïve T cells there is a strong justification for investigating their activity in type 1 diabetes. Until relatively recently, however, opportunities to study DCs in a human disease setting were limited. In recent years there has been an increasing recognition that two of the major DC subsets, the myeloid (mDC) and plasmacytoid (pDC) forms are present at low levels in the circulation, and can be identified by their expression of distinct lineage and functional markers (7; 8). Plasmacytoid DCs, also known as the Type I interferon (IFN)-producing cells, are of particular interest, being specialized in the sensing of virus infection through selective expression of Toll-like receptors (TLRs) specific for viral single stranded RNA (TLR7) and double-stranded DNA (TLR9) (9). Ligation of such viral receptors results in the rapid secretion of Type I IFNs, such as IFN-α, at levels 100-1000 times more than any other cell type. Serum IFN-α levels are elevated in children at diagnosis of type 1 diabetes (10); IFN mRNA subtypes are found in post mortem pancreas samples from type 1 diabetes patients (11); and, critically, IFN-α treatment for diseases such as chronic viral hepatitis and cancer has precipitated the clinical manifestation of autoimmune disease, including type 1 diabetes, in a number of cases (12; 13). Moreover, there is emerging evidence of a close relationship between pDCs, excessive amounts of Type I IFNs and other autoimmune conditions (14).

We hypothesised that the existence of a relationship between Type I IFNs and type 1 diabetes near to the onset of the disease might be reflected in a disturbance in blood DC subsets. Our study demonstrates a profound disturbance in the normal balance of pDCs and mDCs in peripheral blood in the immediate period after diagnosis. Moreover, we show that pDCs capture islet cell autoantigens via immune complexes, and in so doing enhance CD4 T cell activation, suggesting synergy between elements of the innate and adaptive immune systems, and islet cell autoantibodies in particular, in type 1 diabetes-related autoimmunity.

MATERIALS AND METHODS

Subjects. For DC enumeration studies, fresh heparinized blood was obtained from 40 patients with new-onset type 1 diabetes (mean age 27 years ± 7.2; disease duration 30 days ± 15) and 40 non-diabetic control subjects of similar age, sex and HLA type with no family history of autoimmune disease (mean age 29 years ± 5.2). Eighteen patients with long...
standing type 1 diabetes (median disease duration 19.5 years, range 3 to 58 years; mean age 44 years ± 13.8) were also studied along with an additional, appropriately matched non-diabetic control group (n=18; mean age 34 years ± 10). In addition, 16 patients with type 2 diabetes (median disease duration 7.5 years, range 2-16, mean age 59.8 years ± 11.7, were also studied along with an appropriately matched non-diabetic control group (n= 16 mean age years 51 years ± 8.7). Blood was also obtained from a further group of patients with new-onset type 1 diabetes and from control subjects of similar age, sex and HLA-type for detailed analysis of DC phenotype (n=13 in each group) and DC cytokine production after in vitro stimulation (n=9 in each group). All T1D patients gave a history of acute onset of symptoms typical of diabetes and required insulin from diagnosis. Typically, blood from a patient was analyzed alongside that of an appropriate control taken at the same time of day, to avoid any potential confounding effects of diurnal rhythm and season. Patients or control subjects reporting recent (up to 2 weeks) symptoms of “virus-like” illnesses were excluded from participation in the study. Metabolic control was assessed by measurement of HbA1c at the time of blood sampling for DC studies. The presence of autoantibodies to IA-2 and glutamic acid decarboxylase-65 (GAD65) was detected by radio-immunoassay (RSR Ltd, Cardiff, UK).

DC enumeration. Lysed whole blood direct immunofluorescence staining for the quantitation of DC subsets was performed using the Blood DC Enumeration Kit (Miltenyi Biotec Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Briefly, 300 μl of whole blood was incubated with either the anti-BDCA cocktail, containing monoclonal antibodies (Mabs) specific for the DC markers BDCA-1 (CD1c for detecting mDC1), BDCA-2 (CD303 for detecting pDCs) and BDCA-3 (CD141 for detecting mDC2) as well as anti-CD14 (monocytes) and anti-CD19 (B lymphocytes), or the control cocktail (containing appropriate isotype control Mabs). Following 10 minutes photo-incubation with these and a dead cell discriminator on ice, erythrocytes were lysed and remaining cells were washed in PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich, Poole, UK), 0.1% sodium azide, 2% foetal calf serum (FCS, Invitrogen, Paisley, UK) and 1% human AB serum (PAA, Linz, Austria) (flow buffer). Cells were fixed and analysed by four-colour flow cytometry using a FACSCalibur cytometer (BD Biosciences, San Jose, USA). CD14+ and CD19+ and dead cells were excluded and DC subsets enumerated. A minimum of 3.5 x 10⁵ events in the mononuclear (forward and side scatter) gate were collected, of which a mean of 0.8% are DCs (~2800 events). Gates denoting positivity for markers of mDC1, mDC2 and pDC were set using the appropriate isotype control Mabs and the number of DCs in a sample expressed as a percentage of the total number of gated mononuclear cells or the total number of DCs.

Dendritic cell isolation, phenotypic analysis and stimulation. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood by density gradient centrifugation (Lymphoprep; Axis Shield, Dundee, UK). CD14+ and CD19+ cells were depleted from PBMCs using magnetic cell sorting technology (AutoMACS; Miltenyi Biotech). Myeloid DCs were then positively isolated from the remaining PBMCs using BDCA-1⁺ conjugated magnetic microbeads and pDCs then isolated from the remaining cells using BDCA-4⁺ conjugated magnetic microbeads. Averaging from 20 separations, mean (±SD) purity was 78.8% ±10.9 for pDCs and 75.0% ±19.5 for mDCs.

Markers of activation and function were analysed by four-colour flow cytometry using 2 x 10⁶ DCs in 100μl FACS buffer along with
the appropriate fluorochrome labelled Mabs. Samples were incubated on ice in the dark for 30 minutes, and then washed twice in ice-cold flow buffer before immediate analysis. The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC) labelled anti-CD19 (clone LT19), anti-HLA-ABC (clone W6/32) and anti-CD83 (clone HB15e) Mabs; phycoerythrin (PE) labelled anti-CD62L (clone FMC46) and anti-CD80 (clone MEM-233) Mabs (all from Serotec, Oxford UK); PE-labelled anti-CD4 (clone RPA-T4), peridinin chlorophyll protein (PerCP) labelled anti-CD14 (clone MφP9), anti-HLA-DR (clone TU36) Mabs; and allophycocyanin (APC) labelled anti-CD3 (clone UCHT1), anti-CD86 (clone 2331FUN-1) (all BD Pharmingen, San Diego, CA) and anti-CD123 (clone AC145) Mabs (Miltenyi Biotec). Antibody concentrations used were based on data supplied by the manufacturers and in-house optimization studies. For these studies, the selected DC subset was always gated using a specific marker (BDCA1 for mDC and CD123 for pDC). Gated cells were then assessed for the mean fluorescence intensity of the specific activation/maturation marker.

In studies on the cytokine potential of isolated blood DC subsets, mDCs and pDCs were resuspended to 2x10^5 per ml in RPMI 1640 supplemented with penicillin, streptomycin and fungizone (Invitrogen) and 10% human AB serum. mDCs were stimulated with lipopolysaccharide (LPS) (Sigma-Aldrich) at concentrations of 0 and 10ng/ml in a total volume of 200µl in duplicate samples. pDCs underwent similar treatment in cultures supplemented with 10ng/ml recombinant human IL-3 (Strathmann Biotec, Hamburg Germany) and were stimulated with unmethylated oligodeoxynucleotides (ODN 2216, a potent TLR-9 ligand and IFN-α secretagogue; Autogen Biocear, Calne UK) at concentrations of 0, 1, 3, 10 and 30µg/ml in a total volume of 200µl in duplicate samples (15; 16). Following 16 hours stimulation supernatants were collected and stored at -80°C for later analysis. IFN-α production in pDC culture supernatants was quantified by specific sandwich ELISA using the IFN-α module set (Bender MedSystems, detection range 8-5000 pg/ml). There are 14 different IFN-α isoforms, of which the IFN-α module set detects the majority, with the exception of B and F. Supernatants from mDC and pDC cultures were analysed using the Beadlyte Human Cytokine Detection kit 3 (Upstate, Lake Placid USA) according to manufacturer’s instructions. Cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNFα and IFNγ were measured (detection range 6.9-5000 pg/ml). Data were analysed on the Luminex 100 instrument system using STarstation software.

**Presentation of autoantigens by dendritic cells.** cDNA encoding human IA2-intracellular portion (IA-2ic) was modified to include a coding sequence for the HLA-DR4 restricted influenza haemagglutinin CD4 T cell epitope (HA307-319), inserted at the N-terminus of IA-2ic, and recombinant IA-2ic-HA307-319 protein prepared as described (17; 18). An HLA-DR4 restricted CD4 T cell clone specific for HA307-319 (JNZ-1) was generated by peptide stimulation of PBMC cultures, followed by flow cytometric sorting of cells stained positive with an HLA-DR4 tetramer loaded with HA 307-319 (provided by Dr G Nepom, Benaroya Research Institute, Seattle). PDCs, mDCs and CD14⁺ monocytes were isolated from HLA-DR4⁺ subjects, resuspended to 5x10^4/ml and 100µl dispensed into 96-well flat-bottomed plates in serum free X-VIVO 15 (Cambrex, Charles City, USA). Cultures were supplemented with serum from T1D patients positive for either IA-2 or GAD65 autoantibodies, or pooled AB (1-30%) and IA-2ic-HA307-319 (10µg/ml). In some experiments, the IgG fraction in IA-2 autoantibody positive serum was depleted by incubation with protein A matrix (Repligen,
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Waltham, MA, USA; binding capacity 18-43 mg/ml) at a 1:1 ratio for 90 minutes at room temperature according to the manufacturer’s instructions. Following centrifugation (15 seconds at 10,000g) unbound serum was carefully removed and IgG depletion confirmed by laser nephelometric analysis of IgG levels on pre- and post adsorption samples. After 24 hours of DC culture with serum under these conditions, JNZ-1 clone cells (10^5/well) were added and 48 hours later IFN-γ levels in supernatants were quantified by ELISA (Immunotools, Friesoythe Germany; detection range 2-500 pg/ml).

**Statistical analysis.** DC subsets, expressed as a percentage of total DCs, were normally distributed in all study groups according to Kolmogorov-Smirnov analysis. Mean levels of DC subsets were therefore compared between study groups using the Student’s t test. Relationships between DC subset numbers and clinical parameters were compared by calculation of Pearson’s correlation coefficient. Differences in distribution were compared by χ^2 analysis. P values of <0.05 were considered significant. Differences in IFN-γ production were compared using Student’s t-tests.

**RESULTS**

**Detection of DC subsets in whole blood.** DC subsets were identified in whole blood using specific Mabs amongst mononuclear cells identified by their physical side and forward scatter properties (Figure 1A) after removal of CD14^+, CD19^+ and dead cells by exclusion gating (Figure 1B). DC numbers were expressed as a percentage of mononuclear cells and DC subsets as a proportion of total DCs. Representative examples of BDCA-1+ (mDCs), BDCA-2+ (pDCs) and BDCA-3+ (mDC2s) cells are shown in Figures 1C-E. The majority of events in the CD14-/CD19-gate in Figure 1B are T cells, with some NK and NKT cells. The relative proportion of this population was not different between T1D patients and controls (data not shown).

**DC subsets in type 1 diabetes patients.** The total number of DCs (i.e. cells expressing BDCA-1, BDCA-2 or BDCA-3) when expressed as a percentage of the total mononuclear cell population was similar in new-onset type 1 diabetes patients and non-diabetic control subjects (Figure 1F).

However, within the total DC population, DC subsets had a different proportional distribution in the two study groups. The mean percentage of DCs belonging to the pDC subset was significantly higher in new-onset type 1 diabetes patients when compared with non-diabetic control subjects (p=0.002; Figure 2A). In contrast, the mean percentage of mDCs was significantly lower in new-onset type 1 diabetes patients compared with the non-diabetic control group (p=0.003; Figure 2B). These findings were similar regardless of whether DC subsets were expressed as a percentage of total DCs or of mononuclear cells. There was no difference between the study groups in the proportion of DCs belonging to the BDCA3^+ mDC2 populations (Figure 2C). Amongst the new-onset group, there was no correlation between percentages of DC subsets and age or duration of diabetes, or HbA1c levels as a marker of metabolic control. The percentage levels of DC subsets were not different between patients with and without islet cell autoantibodies and did not differ between patient subgroups divided according to HLA class II genotype.

In order to assess whether the abnormalities in DC subsets observed in new-onset type 1 diabetes patients could be genetically determined, we also studied patients in whom type 1 diabetes had been diagnosed for a minimum of 3 years (median duration of disease 19.5, maximum 58 years). In these patients, mean levels of total DCs as a percentage of mononuclear cells, and the distribution of DC subsets were similar to
those in age-matched non-diabetic control subjects (Figure 3A-D). We also examined the potential influence of hyperglycaemia per se on DC subsets by analysis of patients with type 2 diabetes with similar levels of HbA1c (mean ±SD 7.9% ±1.9) compared with the new-onset patients (8.73% ±2.1; p=NS). Chronic hyperglycaemia alone could not account for the changes we observed, since proportions of DC subsets did not differ significantly in patients with type 2 diabetes compared with control subjects (Figure 3E-G).

Although the populations of patients with type 1 diabetes (new-onset and long standing) were not closely age-matched, we also compared the distribution of DC subsets between them; this comparison seemed justified in the absence of any relationship between age and DC subset number in the patient and control subject groups. Mean levels of pDCs were higher, and mean levels of mDCs lower, in the new-onset patients (p=0.0013 and p=0.0014, respectively). These data suggested that DC subset balance is disturbed near to disease diagnosis, but gradually normalises. Further evidence for this was obtained by repeat analysis of blood samples on 5 patients tested both at diagnosis and two years later. Three of the five show evidence of declining pDC percentages and four had increased/unchanged percentages of mDCs at year 2 (Figure 4A-B) although with small numbers of subjects these can only be considered indicative trends (p=NS). This contrasts with data from a control individual analysed seven times over the same 2-year period, in whom pDC and mDC subsets were remarkably stable (Figure 4C). Taken together, these data indicate that type 1 diabetes is characterized by an abnormal distribution of DCs, resulting in high levels of pDCs and low levels of mDCs, but that these changes are restricted to the peri-diagnosis period.

**Expression of differentiation and activation markers on pDCs and mDCs in type 1 diabetes patients.** Since the pDC population appeared to be expanded at diagnosis of type 1 diabetes, we next wished to determine whether this expansion was associated with activation of this subset. Blood pDCs expressed high basal levels of HLA molecules (both class I and class II), IL-3 receptor (CD123) and L-selectin (CD62L), but low levels of the DC activation marker CD83 and the co-stimulatory molecules CD80 and CD86, which are typically up-regulated upon activation. mDCs were also found to express high levels of HLA class I and class II molecules. mDCs also expressed detectable levels of CD83, CD80 and CD86, but without any sign of up-regulation of these activation molecules in patients (see supplementary Figure 1). Formal comparison of the levels of expression of these markers in new-onset type 1 diabetes patients and non-diabetic control subjects (n=13 in each group) failed to show any significant differences.

**Cytokine and chemokine production by pDCs and mDCs.** Plasmacytoid DCs are capable of producing very large quantities of Type I IFNs following stimulation and in order to assess their IFN-α producing capacity in type 1 diabetes, cells isolated from the study groups were cultured with a TLR9 ligand and IFN-α secretion measured. pDCs from both new-onset type 1 diabetes patients and non-diabetic control subjects (n=9 in each group) did not produce IFN-α without stimulation. After stimulation with CpG ODN, pDCs from patients and control subjects produced similar, large amounts of IFN-α in a dose dependent manner (Figure 5). Thus, per-pDC IFN-α secretion is not abnormal in patients and there is no evidence of spontaneous ex vivo IFN-α secretion by these cells, making it unlikely that they are the source of the elevated serum levels of this cytokine that has been reported in T1D patients (10).

Using the same samples we also examined basal and stimulated secretion of IL-1β, IL-6,
IL-8, IL-10, IL-12p70, TNFα and IFNγ by pDCs and mDCs. No differences between patients and controls were observed.

**Islet cell autoantibody-positive serum enhances autoantigen presentation to pDCs.** It has recently been reported that pDC presentation of antigen is enhanced in the presence of immune complexes containing the relevant antigen (19). We designed experiments in which pDCs, mDCs and monocytes presented a hybrid construct of IA-2ic containing the influenza haemagglutinin epitope HA307-319. Activation of an HA307-319-specific CD4 T cell clone (JNZ-1) was used as the measure of efficiency of IA-2ic-HA307-319 presentation. Serum positive or negative for IA-2 autoantibodies was then added to examine whether presentation was enhanced in the presence of relevant autoantibody specificities. Presentation of IA-2ic-HA307-319 by pDCs was enhanced in the presence of serum containing IA-2, but not GAD65 autoantibodies (Figure 6A). IFN-γ production by the T cell clone was more than doubled in the presence of IA-2 autoantibodies (p<0.05 compared with control serum for 5% and 10% serum supplementation).

In contrast, mDC and monocyte presentation of IA-2ic-HA307-319 was largely unaffected by the presence of islet cell autoantibodies (Figure 6B-C). In the case of mDCs, it is probable that their uptake and presentation of antigen through pinocytosis is highly efficient in the absence of immune complexes, and therefore not enhanced to any great degree in their presence.

The effect of IA-2+ serum samples in enhancing antigen presentation of IA-2ic-HA307-319 was seen in samples from more than one patient with IA-2 autoantibodies and was highly significant (Figure 6D,E). The enhancing effect was attributable to the IgG fraction in the serum samples, since it was significantly diminished after IgG depletion using a protein A-sepharose column (Figure 6F). Enhanced presentation of IA-2 by pDCs was not associated with IFN-α production.

**DISCUSSION**

Our study shows an abnormal distribution of the main blood DC subsets in Type 1 diabetes. Interferon-producing pDCs, rather than mDCs, predominate proportionally amongst DCs in type 1 diabetes patients, and since these changes were only observed at diagnosis of disease, it seems unlikely that the alterations are genetically determined. The increased proportion of pDCs and reduction in mDCs was not accompanied by changes in activation status of these cells, nor, in the case of pDCs, by a change in their capacity for IFN-α secretion. There is no immediate explanation for this increased representation of pDCs, but taken together with previous reports of abnormal levels of circulating and tissue-deposited Type I interferons in type 1 diabetes, these findings extend the concept of a relationship between Type I interferons, their major cellular source the pDC, and the onset of Type 1 diabetes. Further weight is given to the potential importance of this relationship by our demonstration that pDCs have enhanced ability to activate primed effector CD4 T-cells in the presence of islet cell autoantibodies, indicating a route through which autoantibodies could promote cell-mediated islet autoimmunity.

Reagents for the reliable detection of DC subsets in peripheral blood have only recently become available, and few investigators have yet had the opportunity to deploy this technology in autoimmune diseases. To the best of our knowledge, this is the first study to demonstrate an increase in pDCs in new onset type 1 diabetes patients using this approach. These findings are similar to those in the preliminary report of Peng et al, who did not use the BDCA DC markers, but rather detected pDCs as lineage-negative/HLA-DR+/CD11c-/CD123+ and mDCs as lineage-negative/HLA-DR+/CD11c+/CD123- cells.
This group also found a higher proportion of pDCs in type 1 diabetes, although specific results are not reported, limiting the opportunity for direct comparison. A more recent report focuses on patients with type 1 diabetes of at least 5 years duration, and finds no abnormality of blood DC balance, similar to our own analysis of long-standing patients (21). In contrast, Vuckovic et al have reported decreased DC numbers in children with new onset type 1 diabetes (22). However, these authors did not use specific markers of DC subsets, and report levels of blood DCs (>100 cells/μl) that are vastly in excess of the known concentration of these cells (approximately 1% of PBMCs, equivalent to 10-20 cells/μl (7)).

Using an in vitro culture system, we show that in the presence of islet cell autoantibodies of the relevant specificity, the ability of pDCs to process and present islet autoantigens to CD4 T-cells is significantly enhanced. This immune complex capture is known to operate through the FcγRII-receptor for IgG (CD32), probably through enhanced antigen uptake (19). Antibody-enhanced cross-presentation of an islet antigen has also been shown to break tolerance in a murine model of autoimmune diabetes (23). We consider it unlikely that such a pathway is involved in the initial priming of islet autoimmunity in man, but it could have an important role in the amplification and maintenance of T cell responses, and also in epitope spreading (24). The finding that immune complex capture by pDCs enhances autoreactive T cell activation highlights a potential route through which autoantibodies could participate in cell-mediated autoimmune responses, in addition to that offered by B lymphocyte presentation of autoantigens.

It is unclear why DC balance should be perturbed near to diagnosis of type 1 diabetes, but our studies exclude any possible effects of glycaemic control. One explanation is that selective migration of mDCs into tissues or lymph nodes results in a relative expansion of blood pDCs, especially if accompanied by proliferation of the DC-precursor pool. The possible effect of DC migration on blood levels has been noted in diseases in which both inflamed tissues and the circulation have been examined, such as psoriasis (25), systemic lupus erythematosus (26) and respiratory syncytial virus infection (27), each of which are associated with a reduction in pDCs in peripheral blood and infiltration of the tissues. An alternative hypothesis is that pDC expansion occurs in response to a virus infection that arises towards the end of the islet destructive process. Virus infection at this late stage of disease is known to be capable of accelerating disease onset in animal models (28), and it is tempting to speculate that this induces a shift in the ratio of plasmacytoid and myeloid dendritic cells in the periphery.

At this stage it is only possible to speculate on the pathological relevance of our findings.

Whilst the possibility of a pro-inflammatory role is attractive, studies in animal models show that an increase in pDC/mDC ratio may prevent antigen-specific inflammatory responses, including diabetes (29-31), and a similar role in type 1 diabetes cannot be excluded. Future studies that examine whether DC subsets are disturbed in the prediabetic period and therefore reflect events involved in the chronic autoimmune process of islet destruction, or whether pDC expansion is related solely to peri-diagnosis events, may clarify this.

**ACKNOWLEDGEMENTS**

This work was supported by a grant from Diabetes UK. The authors acknowledge financial support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership
with King's College London. We are grateful to Dr G Nepom for supply of the HA\textsubscript{307-319}–DR4 tetramer.
Figure 1. Example of flow cytometry analysis and gating strategy for identification and quantification of blood DC subsets. The mononuclear cell population in whole blood is identified by a combination of forward and side scatter properties (A) followed by exclusion gating of live CD14+ and CD19+ mononuclear cells and dead cells (B), before the identification and enumeration of pDC, mDC1 and mDC2 subsets as shown in panels C-E with the relevant blood dendritic cell (BDC) marker. Total DC populations as a percentage of mononuclear cells in 40 patients with new onset type 1 diabetes (T1D) and matched control subjects are shown in panel F.

Figure 2. Dendritic cell subsets in new-onset type 1 diabetes (T1D) patients and controls, showing plasmacytoid DCs (A), type 1 myeloid DCs (B) and type 2 myeloid DCs (C). Results are expressed as % of total DC population. Mean values for each group are indicated by a horizontal line and p values given where significant.
Figure 3. Dendritic cell subsets in long standing type 1 diabetes patients (LS T1D) and age-matched control subjects, showing total DC population as a percentage of mononuclear cells (A) and the plasmacytoid DC (B), type 1 myeloid DC (C) and type 2 myeloid DC (D) subsets as a percentage of total DCs. Mean values for each group are indicated by a horizontal line, and were similar between study groups. Similarly panels E-G show plasmacytoid DC (E), type 1 myeloid DC (F) and type 2 myeloid DC (G) subsets as a percentage of total DCs in patients with type 2 diabetes and an appropriately age-matched control group. Mean levels are similar in the two groups (p=NS).
Figure 4. (A) Plasmacytoid and (B) myeloid dendritic cell subsets as a percentage of total DCs in 5 patients with new onset type 1 diabetes measured at diagnosis and two years later. There is a trend in three of the patients for pDC levels to decline and in three of the patients for mDC levels to increase/remain unchanged. (C) DC subset levels (expressed as a percentage of DCs) in a non-diabetic control individual studied on seven separate occasions over the same 2 year period.
Figure 5. IFN-α secretion by isolated pDCs in type 1 diabetes patients and control subjects in response to stimulation with increasing concentrations of the unmethylated CpG oligonucleotide 2216. Each point represents the mean value of IFN-α secretion from 9 different patients (closed triangles) and 9 control subjects (open squares). Vertical bars represent SEMs.

![Graph showing IFN-α production vs CpG concentration](image)

Figure 6. Effect of islet cell autoantibody-positive serum on presentation by pDCs, mDCs and CD14+ monocytes of the islet cell autoantigen IA-2ic-HA_{307-319} to CD4 T cells (JNZ-1 clone) specific for the influenza HA_{307-319} epitope. CD4 T cell clone responses are indicated by levels of IFN-γ produced in the culture supernatants. In the presence of soluble 10μg/ml IA-2ic-HA_{307-319} without serum supplementation (open bars), IA-2ic is processed and presented with comparable efficiency by pDCs and mDCs, with CD14+ monocytes markedly less proficient. Addition of IA-2 autoantibody-positive serum (3500 WHO units) at 1, 5 and 10% considerably and significantly enhances the presentation of IA-2ic by pDCs but has minimal effects on mDCs and CD14+ monocytes (*indicates p<0.05 versus presence of antigen alone (open bars). GAD-65 autoantibody-positive serum (1700 WHO units; grey bars; 1-10%) has no effect on presentation. Likewise, pooled AB serum at these concentrations has no effect (data not shown). In the presence of 5% serum but no IA-2ic-HA_{307-319} there is no T cell activation. Data represent one of 4 experiments using 4 different cell donors. Panel D shows pooled data from a total of 4 donors (including the one used in panel A) for pDC enhancement of IA-2ic-HA_{307-319} presentation to JNZ-1 clone cells (*indicates p<0.05, **indicates p<0.01 and ***p<0.001 versus presence of antigen alone (open bars). The additional sera contained 3875.1, 3263.1 and 3007.8 WHO units of IA-2 autoantibodies. Bars represent mean (SEMs) from quadruplicate wells of individual experiments. Panel E shows that IA-2+ serum enhanced IA-2ic-HA_{307-319} presentation is dependent upon the serum IgG fraction. Serum has been adsorbed using Protein A matrix. Serum IgG level pre-adsorption 6.54g/l; after adsorption 0.22g/l. Serum was used at 5% concentration in these experiments. Serum IA-2 autoantibody level pre-adsorption 2152 WHO units; after adsorption IA-2 autoantibodies were not detectable. * indicates a significant reduction in IFN-γ.
production by clone JNZ-1 in the presence of adsorbed, compared with non-adsorbed serum (p<0.05).

![Graphs showing IFNγ production by different cell types: pDCs, mDCs, CD14+ monocytes, and IA-2+ serum with varying concentrations of antigen.]

- **A**: pDCs production in the presence of adsorbed vs. non-adsorbed serum.
- **B**: mDCs production with IA-2 AAb+ serum.
- **C**: CD14+ monocytes with IA-2 AAb+ serum.
- **D**: IFNγ production with IA-2 serum and varying antigen concentrations.
- **E**: IFNγ production with IA-2+ serum and IA-2 (pre-adsorbed).
REFERENCES