Dysregulated Toll-Like Receptor–Induced Interleukin-1β and Interleukin-6 Responses in Subjects at Risk for the Development of Type 1 Diabetes

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We tested the hypothesis that altered Toll-like receptor (TLR) signaling may be involved in early stages of type 1 diabetes (T1D). To do so, we analyzed TLR-induced interleukin (IL)-1β and IL-6 responses in freshly isolated peripheral blood mononuclear cells (PBMNCs) from seropositive compared with seronegative subjects. Similar frequencies of myeloid dendritic cells (mDCs), plasmacytoid DCs (pDCs), and monocytes were observed in seropositive and seronegative subjects. Subjects with autoantibodies had increased proportions of monocytes expressing IL-1β ex vivo. Activating PBMNCs with TLR3, TLR4, or TLR7/8 agonists in vitro led to increased percentages of IL-1β–expressing monocytes and mDCs from seropositive versus seronegative subjects. TLR ligation also resulted in a diminished IL-6 response in seropositive individuals as lower frequencies of IL-6–expressing monocytes and mDCs were induced. The dysregulated TLR-induced IL-1β and IL-6 pathways were more readily detectable in children aged <11 years and from 11 to <21 years, respectively, and did not involve altered HbA_{1c} or the presence of one or more autoantibodies. Finally, subjects with autoantibodies had lower amounts of serum chemokine (C-X-C motif) ligand 10 compared with autoantibody-negative subjects. Our data may imply that alterations in innate immune pathways are detectable in genetically susceptible individuals and could be linked with the early course of T1D. ***Diabetes*** **61:2525–2533, 2012**

Type 1 diabetes (T1D) is an autoimmune disease that involves the progressive destruction of insulin-producing cells (1). How the disease is triggered is unknown; however, studies in animal models of T1D support the hypothesis that microbial infections and the innate immune system play a key role in disease mechanisms (2).

The appearance of autoantibodies against islet antigens is one of the early manifestations of autoimmune destruction of pancreatic islet β-cells. It is now possible to predict the development of T1D based on a number of risk factors, including the presence of autoantibodies against insulin, GAD65, insulinoma-associated protein-2 (IA-2), and zinc transporter 8 (ZnT8) (3) that can, in most cases, be found in the peripheral blood many years before the onset of hyperglycemia (1). Insulin-specific autoantibodies are typically the first to be expressed, particularly in young children, as reviewed by Barker (4). The 5-year risk of diabetes is 75% in family members who express two or more of the islet autoantibodies compared with 25% in relatives who express only one of those autoantibodies (5).

Toll-like receptors (TLRs) expressed on dendritic cells (DCs) and macrophages play a major role in host defense against invading pathogens. TLRs identify microbes via a limited number of germline-encoded receptors, termed pattern recognition receptors (PRRs), that recognize highly conserved molecular structures expressed by viruses, bacteria, and fungi, termed pathogen-associated molecular patterns (PAMPs) (6–10). PAMP binding by PRRs induces a cascade of intracellular signaling events that lead to upregulation of antimicrobial genes, proinflammatory cytokines and chemokines, and major histocompatibility complex class II and costimulatory molecules on the cell surface membrane, facilitating the ability of antigen-presenting cells (APCs) to efficiently present antigens to T cells (6–10).

We recently found that activation of the TLR4 pathway in newly diagnosed patients with T1D induced a higher frequency of interleukin (IL)-1β–expressing monocytes and reduced percentages of IL-6–expressing myeloid dendritic cells (mDCs) compared with nondiabetic individuals (11). We hypothesized that these alterations may be associated with early disease mechanisms. In the current study, we tested the hypothesis that altered innate immunity is also detectable in genetically susceptible individuals before disease onset. To this end, we analyzed TLR-induced IL-1β and IL-6 production by monocytes and mDCs from subjects positive for islet autoantibodies enrolled in the Type 1 Diabetes TrialNet Natural History study and the Diabetes Autoimmunity Study in the Young (DAISY). We provide evidence that dysregulated TLR-induced IL-1β and IL-6 cytokine responses exist in the peripheral blood from autoantibody-positive subjects compared with seronegative individuals. We hypothesize that these alterations are involved in the early course of T1D.

RESEARCH DESIGN AND METHODS

Study participants. Blood samples were drawn from islet autoantibody-positive and -negative subjects enrolled in the Type 1 Diabetes TrialNet Natural History (12) and DAISY (13) studies during their routine study visits. Both are prospective studies conducted in first-degree relatives of patients with T1D with the goal of better understanding the natural history of the disease (12,13). Subjects were asked about their health status, including recent infections. Only individuals free of a known infection at the time of the blood draw were included in this study. Sera and peripheral blood mononuclear cells (PBMNCs) were isolated immediately after the blood had been drawn. The study was approved by the institutional review board at the University of Colorado Denver.

Because of the age of some participants when sampled, some samples were limited in volume for testing and complete analysis was not possible. The presence of autoantibodies to GAD65, IA-2, and insulin in the serum was determined by the Diabetes and Endocrinology Research Center Autoantibody Core Facility at the Barbara Davis Center for Childhood Diabetes, University of Colorado Denver. The autoantibody-positive cohort used for our TLR-induced

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cytokine response included 66 subjects (35 females and 31 males; average age 21.4 ± 13.5 years, range 4–53), with one or more islet autoantibodies. Twenty subjects were included in the group aged <11 years (average 9.0 ± 1.4), 21 in the group aged from 11 to <21 years (average 15.3 ± 2.8), and 25 in the group aged ≥21 years (average 36.5 ± 9.4). Of these, 44 subjects were positive for one autoantibody, 20 were positive for two autoantibodies, and 2 were positive for three autoantibodies. The median HbA_{1c} value in the autoantibody-positive group was 5.2 (range 4.5–6.2). The control group included 53 autoantibody-negative individuals (29 females and 24 males; average age 13.0 ± 8.3 years, range 6–54); 24 subjects were included in the group aged <11 years (average 8.3 ± 1.4), 24 in the group aged from 11 to <21 years (average 13.0 ± 1.4), and 5 were included in the group aged ≥21 years (average 37.5 ± 14.4).

The study cohort used for our serum cytokine measurement studies included 40 subjects (26 females and 14 males; average age 22.5 ± 15.6 years, range 5–53), with one or more islet autoantibodies. The median HbA_{1c} value in the autoantibody-positive group was 5.3 (range 4.5–6). Of these, 30 were positive for one autoantibody, 8 were positive for two autoantibodies, and 2 were positive for three autoantibodies. The control group included 40 autoantibody-negative individuals (21 females and 19 males) who were an average age of 14.0 ± 9.2 years (range 5–43).

PBMNC isolation. PBMNCs were freshly isolated by Ficoll-Hypaque Plus density centrifugation (GE Healthcare, Uppsala, Sweden) of freshly drawn heparinized blood from autoantibody-positive and autoantibody-negative subjects. PBMNCs were washed twice with PBS (Invitrogen Life Technologies) and resuspended in endotoxin-free high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 2 mmol/L L-glutamine and 100 units/mL penicillin/streptomycin (both from Invitrogen Life Technologies), and 10% human AB serum (PAA Laboratories, New Bedford, MA). For flow cytometry analyses, PBMNCs were washed and resuspended in fluorescence-activated cell sorter buffer consisting of PBS (Invitrogen Life Technologies) plus 1% BSA and 0.05% sodium azide (both from Sigma-Aldrich).

Analysis of DC and monocyte frequencies in the peripheral blood. PBMNCs were isolated from freshly drawn blood as described above. For mDC enumeration, we used APC-conjugated anti-cluster of differentiation 1 (CD1) C (mouse IgG2a, clone AD5-8E7, Miltenyi Biotech) plus APC-Alexa Fluor 750-conjugated anti-CD19 (mouse IgG1, clone HIB19, eBioscience, San Diego, CA). Staining with anti-CD19 was performed to exclude B cells expressing CD1C. For monocyte staining, we used pacific blue–conjugated anti-CD14 monoclonal antibody (mAb; mouse IgG2a, clone M5E2, BioLegend, San Diego, CA). For plasmacytoid dendritic cell (pDC) subset staining, cells were surface stained with phycoerythrin-conjugated mAb directed against $CD304^+$ (mouse IgG1, clone AD5-17F6, Miltenyi Biotech, Auburn, CA).

Activation of PBMNCs with TLR ligands and intracellular cytokine and chemokine staining. PBMNCs were added to a 96-well round-bottom microtiter plates at a concentration of 1×10^6/well in a total volume of 100 μL. For intracellular cytokine analysis, PBMNCs were incubated in the presence or absence of various purified TLR ligands and 1 μL/mL brefeldin A (BD Biosciences, San Diego, CA) for 4 h, followed by staining for surface markers and intracellular cytokines. For intracellular cytokine staining, PBMNCs were cultured in the presence or absence of 100 ng/mL ultrapurified lipopolysaccharide (LPS; O111:B4; InvivoGen, San Diego, CA), 50 μg/mL polyinosinic:polycytidylic acid (Poly[I:C]; Sigma-Aldrich, St. Louis, MO), and 10 μg/mL R848 (Axxora, San Diego, CA) for 4 h, followed by staining for DC and monocyte surface markers, followed by staining for IL-1β and IL-6, as previously published (11). All TLR ligands were dissolved in Dulbecco's PBS and stored in aliquots at –20°C until used.

Serum cytokine expression levels. Twenty-five microliters of serum were used for measuring levels of IL-1 receptor antagonist (IL-1RA), interferon (IFN)-α, IFN-γ, IL-1α, IL-1β, IL-6, IL-17A, chemokine (C-X-C motif) ligand (CXCL-10), and monocyte chemoattractant protein (MCP)-1 using the Meso Scale Discovery (MSD) cytokine multiplex kit according to the manufacturer's instruction (MSD, Gaithersburg, MD). The detection sensitivity of the MSD method is considered to be greater than other systems (14,15). The diluent included in the kit contains blockers for the neutralization of heterophilic antibodies present in human serum. We measured levels of IL-1 receptor antagonist (IL-1RA), IL-1α, IL-1β, IFN-α2, IFN- γ, IL-6, IL-17A, CXCL-10, and MCP-1. The detection sensitivity limits for these cytokines are 120, 0.12, 0.3, 0.7, 1, 0.7, 0.4, and 3.5 pg/mL, respectively. The diluent MSD plates were read using the MSD SECTOR Imager 2400.

Statistical analyses. Statistical differences in frequencies of freshly drawn mDCs, pDCs, and monocytes, as well as frequencies of monocytes and mDCs–expressing cytokines after TLR ligation in positive versus negative subjects, frequencies of monocytes and mDCs expressing cytokines in at-risk subjects positive for one versus more than one autoantibodies, cytokine responses based on age group, cytokine expression levels, and serum cytokine levels in subjects with one versus more than one autoantibodies were evaluated using the nonparametric Mann-Whitney *U* test. Associations between cell frequencies or cytokine levels and age were evaluated using Pearson correlation analysis. Age correction and ANOVA were applied when a significant association was observed. For statistical differences in serum cytokine levels between seropositive and seronegative subjects and to correct for the effect of age, cytokine concentrations were log-transformed to obtain approximately normally distributed measures and then analyzed using ANOVA. Values of $P < 0.05$ were considered to be statistically significant. Analyses were performed in SAS 9.2 software (SAS Institute, Cary, NC).

RESULTS

DC and monocyte proportions in the peripheral blood. We tested the possibility that altered frequencies of DC subsets and monocytes in the peripheral blood may be involved in the early course of T1D in seropositive versus seronegative subjects before disease onset. PBMNCs were isolated from blood samples drawn as described in RESEARCH DESIGN AND METHODS. The percentages of mDCs, pDCs, and monocytes from subjects positive for autoantibodies were slightly elevated compared with individuals negative for autoantibodies (Fig. 1*A* and *B*); however, this difference did not reach a statistically significant level. The data imply that the frequency of peripheral monocytes and DC subsets is not altered in seropositive subjects at risk for T1D.

TLR-induced IL-1β–expressing monocytes and mDCs. The IL-1 pathway has previously been implicated in humans with T1D (16–19) as well as in animal models of the disease (20). We recently reported that monocytes and mDCs from newly diagnosed patients with T1D have an altered TLR-induced IL-1 pathway (11). We tested the hypothesis that this alteration is detectable in peripheral monocytes and mDCs from nondiabetic individuals positive for one or more anti-islet autoantibodies. To test this, we used flow cytometry to determine the proportion of IL-1β–producing monocytes and mDCs after TLR ligation in vitro. Results show that 13.9 ± 19.6% of monocytes from autoantibody-positive subjects produced IL-1β ex vivo compared with only 4.3 ± 5.0% in autoantibody-negative control individuals ($P = 0.003$; Fig. 2*A* and Supplementary Fig. 1). Stimulation of PBMNCs with LPS led to IL-1β expression in 87.1 ± 11.9% of total monocytes in autoantibody-positive, compared with 79.5 ± 11.8% in autoantibody-negative subjects ($P < 0.01$; Fig. 2*A* and Supplementary Fig. 1). Similarly, activation of the TLR3 and TLR7/8 pathways resulted in an increase of ~10% in the proportion of IL-1β–expressing monocytes from autoantibody-positive individuals versus those without autoantibodies ($P = 0.008$ and $P = 0.002$, respectively; Fig. 2*A* and Supplementary Fig. 1).

Next, we assessed whether mDCs from autoantibody-positive individuals also have dysregulated IL-1β responses. We found that TLR7/8 ligation led to a moderate increase of 7% in the proportion of IL-1β–expressing mDCs from autoantibody-positive subjects versus the control individuals ($P = 0.01$; Fig. 2*B* and Supplementary Fig. 1). We further evaluated whether the magnitude of the TLR responsiveness in seropositive subjects is associated with the number of autoantibodies. Similar frequencies of cells expressing IL-1β in monocytes and mDCs were detectable in individuals positive for one versus two and three autoantibodies (data not shown, $P > 0.05$). Taken together, our observations indicate that alterations in TLR-induced IL-1β pathways are detectable in monocytes and mDCs from seropositive subjects before disease onset.

Correlation between TLR-induced IL-1β responses and age. We checked whether the altered IL-1β responses observed in monocytes from seropositive subjects correspond to any specific age groups. To test this, we analyzed

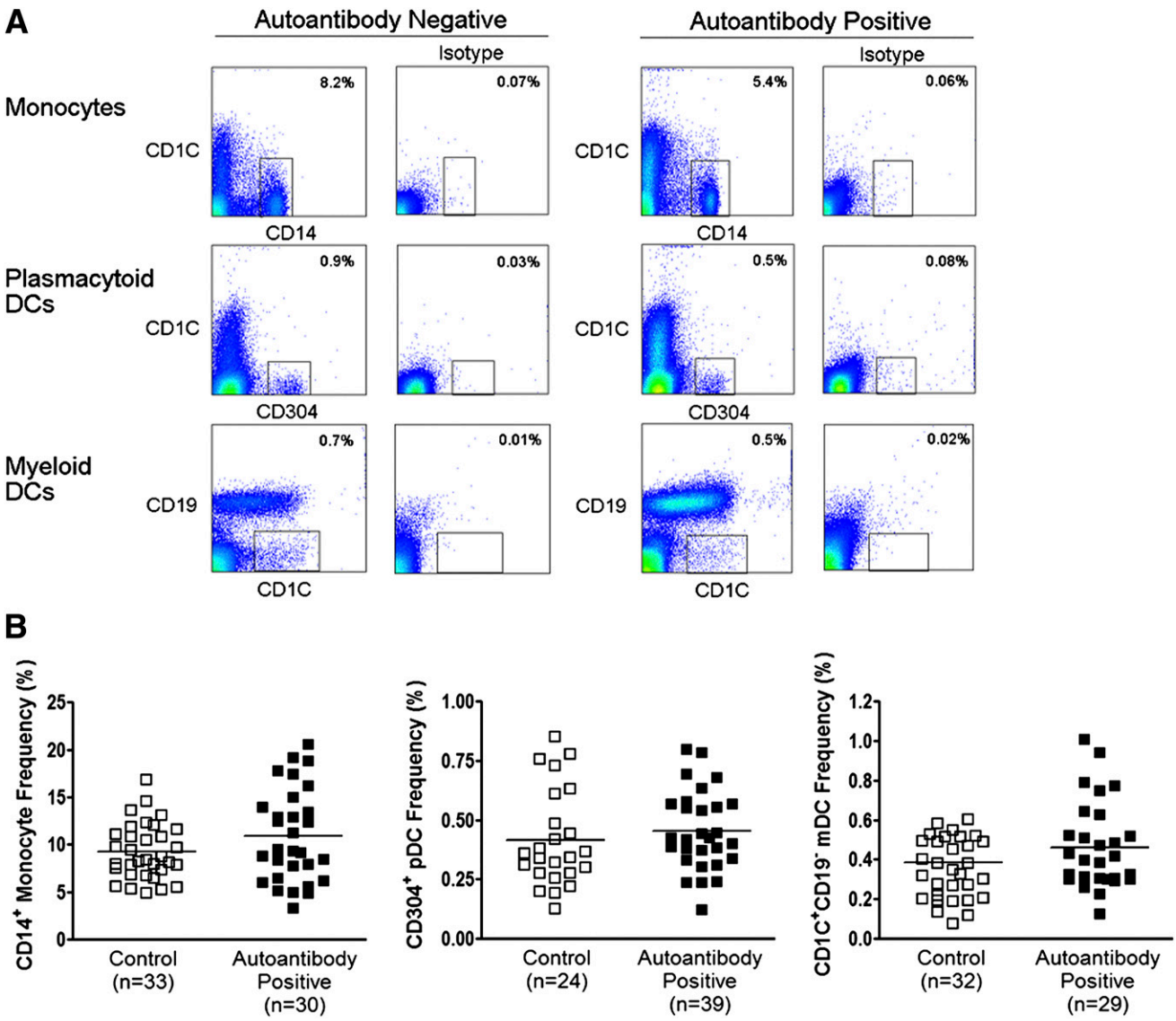

FIG. 1. Frequencies of monocytes and DC subsets in the peripheral blood. PBMNCs isolated from the blood of autoantibody-positive subjects enrolled in the Type 1 Diabetes TrialNet and DAISY studies and autoantibody-negative control subjects stained for surface markers characteristic of monocytes, mDCs, pDCs, or the appropriate isotype control. *A*: Representative flow images show stained monocytes and DCs from autoantibody-negative vs. autoantibody-positive subjects. *B*: Frequencies are shown of monocytes and DC subsets from seronegative vs. seropositive subjects. The results are expressed as percentages of cell subtypes from the total PBMNCs. The bars indicate the mean values. (A high-quality color representation of this figure is available in the online issue.)

IL-1β responses in subjects stratified into three age groups: <11 years (young children), from 11 to <20 years (adolescents), and ≥21 years (adults). The data shown in the upper panel of Fig. 3*A* demonstrate that the presence of monocytes expressing IL-1β ex vivo was more substantial in individuals aged ≥21 years. Data presented in Fig. 3*A* also show that increased frequencies of monocytes expressing IL-1β after LPS activation were more readily detectable in seropositive subjects from the group aged <11 years, as evident by a difference of ~12% in the proportion of IL-1β–expressing monocytes from autoantibody-positive compared with autoantibody-negative subjects ($P = 0.005$). Similar findings were seen in TLR3 and TLR7/8 activated monocytes from seropositive individuals aged <11 years. Increased IL-1β responses were also more readily detectable in mDCs from children aged <11 years after TLR4 and TLR7/8 activation (Fig. 3*B* and data not shown). These data may indicate that altered IL-1β responses are more prevalent in monocytes and mDCs from seropositive young children aged <11 years.

TLR-induced IL-6–expressing monocytes and mDCs. We next assessed whether alterations in the TLR-induced IL-6 pathway are involved in the early course of T1D before hyperglycemia, as observed in new-onset patients with T1D (11). We observed that PBMNC activation with TLR3, TLR4, and TLR7/8 ligands resulted in a modest reduction in the percentage of IL-6–expressing monocytes from autoantibody-positive versus seronegative individuals (Fig. 4*A* and Supplementary Fig. 2). A reduction of ~11% was observed in PBMNCs activated with Poly(I:C) and R848 ($P = 0.02$ and $P = 0.004$, respectively; Fig. 4*A* and Supplementary Fig. 2). No statistically significant differences in the frequency of IL-6–expressing mDCs were observed between seropositive and seronegative individuals (Fig. 4*B*). We further checked whether the level of TLR-induced IL-6–expressing monocytes and mDCs in

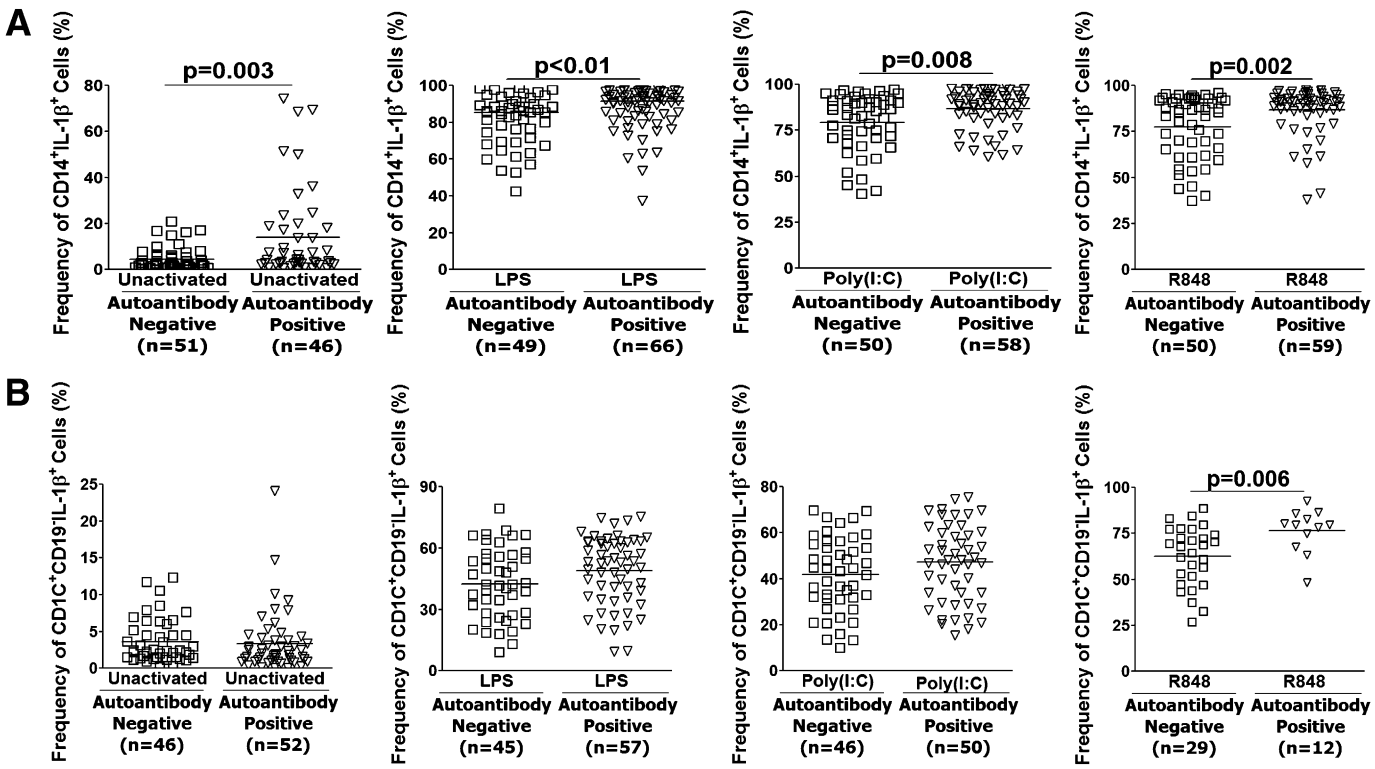

FIG. 2. TLR-induced IL-1β expression in monocytes and mDCs. PBMNCs were unactivated or activated with purified LPS, Poly(I:C), and R848 in the presence of brefeldin A, followed by staining with fluorochrome-conjugated mAbs directed against surface markers for monocytes ($CD14^+$) and mDCs ($CD1C^+CD19^-$). The cells were fixed, permeabilized, and labeled with fluorochrome-conjugated mAbs against IL-1β or the appropriate isotype controls. Each symbol represents an individual percentage of monocytes (*A*), or mDCs (*B*) expressing IL-1β from the total monocytes and mDCs, respectively. The bars indicate mean values. *P* values were evaluated using the nonparametric Mann-Whitney *U* test.

seropositive subjects is influenced by the number of autoantibodies. The frequencies of IL-6–expressing cells did not correspond with the number of autoantibodies (data not shown, $P > 0.05$). Taken together, the data indicate that alterations in the IL-6 pathway may occur in seropositive subjects before disease onset.

Correlation between TLR-induced IL-6 and age. We next checked whether the TLR-induced IL-6 response in monocytes is linked with a specific age group. Analysis of the IL-6 response demonstrated that altered IL-6 responses were more readily detectable in monocytes from autoantibody-positive subjects aged from 11 to <21 years after ligation of TLR7/8, as evident by a difference of 20% between seropositive versus seronegative individuals ($P = 0.008$; Fig. 5*A*). Similar observations were made in Poly(I:C)-activated monocytes from seropositive individuals at age 11 to <20 years (data not shown). Results presented in Fig. 5*B* demonstrate that reduced frequencies of mDCs expressing IL-6 were more readily detectable in the group aged from 11 to <20 years after TLR7/8 ligation, similar to the results observed in monocytes. On the basis of our data, we hypothesize that reduced TLR-induced IL-6 responses may be more prevalent in monocytes and mDCs from 11- to <20-year-old seropositive adolescents.

Serum cytokine and chemokine levels. We used the MSD approach to analyze the level of IL-1RA, IFN-α, IFN-γ, IL-1α, IL-1β, IL-6, IL-17A, CXCL-10, and MCP-1 in sera from seropositive versus seronegative subjects. These cytokines and chemokines have previously been implicated in the course of T1D in humans and animal models (11). IL-6, MCP-1, and CXCL-10 were detected in sera from all subjects, IFN-γ and IL-17A were detected in most of the samples, and the other cytokines were not detectable in most of the samples. Interestingly, we found that the level of none of the cytokines and chemokines tested was increased in seropositive versus seronegative subjects (Fig. 6*A* and data not shown). Moreover, a reduction of more than twofold in the level of CXCL-10 was observed in sera from autoantibody-positive versus autoantibody-negative individuals ($P = 0.01$; Fig. 6*A*). The level of CXCL-10 did not correlate with any specific age group (data not shown).

We further assessed whether serum cytokine and chemokine levels correspond to the number of autoantibodies present. Subjects were stratified by the expression of one or more than one autoantibody. Results presented in Fig. 6*B* indicate that individuals with more than one autoantibody had significantly lower amounts of CXCL-10 than subjects with only one autoantibody ($P = 0.01$). These data imply that altered CXCL-10 serum level could be involved in the early course of T1D in seropositive individuals and that this may be associated with the number of anti-islet antibodies present in the peripheral blood.

DISCUSSION

We analyzed TLR responsiveness in PBMNCs from seropositive individuals and compared it with seronegative subjects. We document for the first time that freshly isolated monocytes and mDCs from autoantibody-positive subjects have dysregulated TLR-induced IL-1β and IL-6 pathways compared with autoantibody-negative individuals via mechanisms that may be linked with age but not

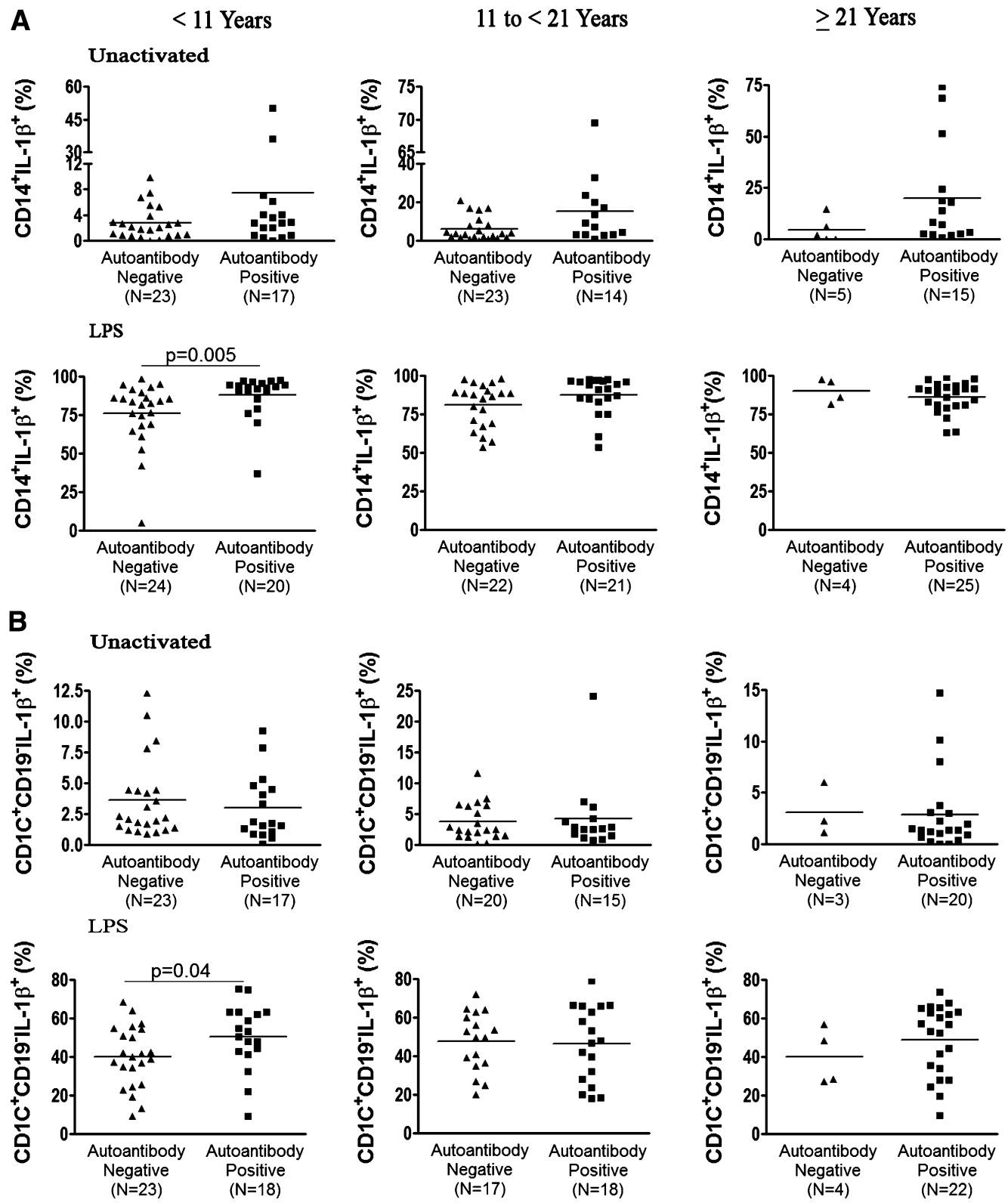

FIG. 3. Correlation between TLR-induced IL-1β in monocytes and mDCs and age. IL-1β responses were evaluated in subjects stratified into three age groups: <11 years (young children), from 11 to <21 years (adolescents), and ≥21 years (adults). Each symbol represents an individual frequency of IL-1β–expressing monocytes (*A*) and mDCs (*B*) after activation with LPS. The bars represent the mean values. *P* values were evaluated using the nonparametric Mann-Whitney *U* test.

with altered proportions of peripheral monocytes and DCs, aberrant levels of HbA_{1c}, or the presence of one versus more than one autoantibody. We also document that autoantibody-positive subjects have lower levels of CXCL-10 in their blood. The observation that altered innate immunity is detectable in at-risk subjects before disease onset supports the hypothesis that this alteration may be linked with early disease mechanisms.

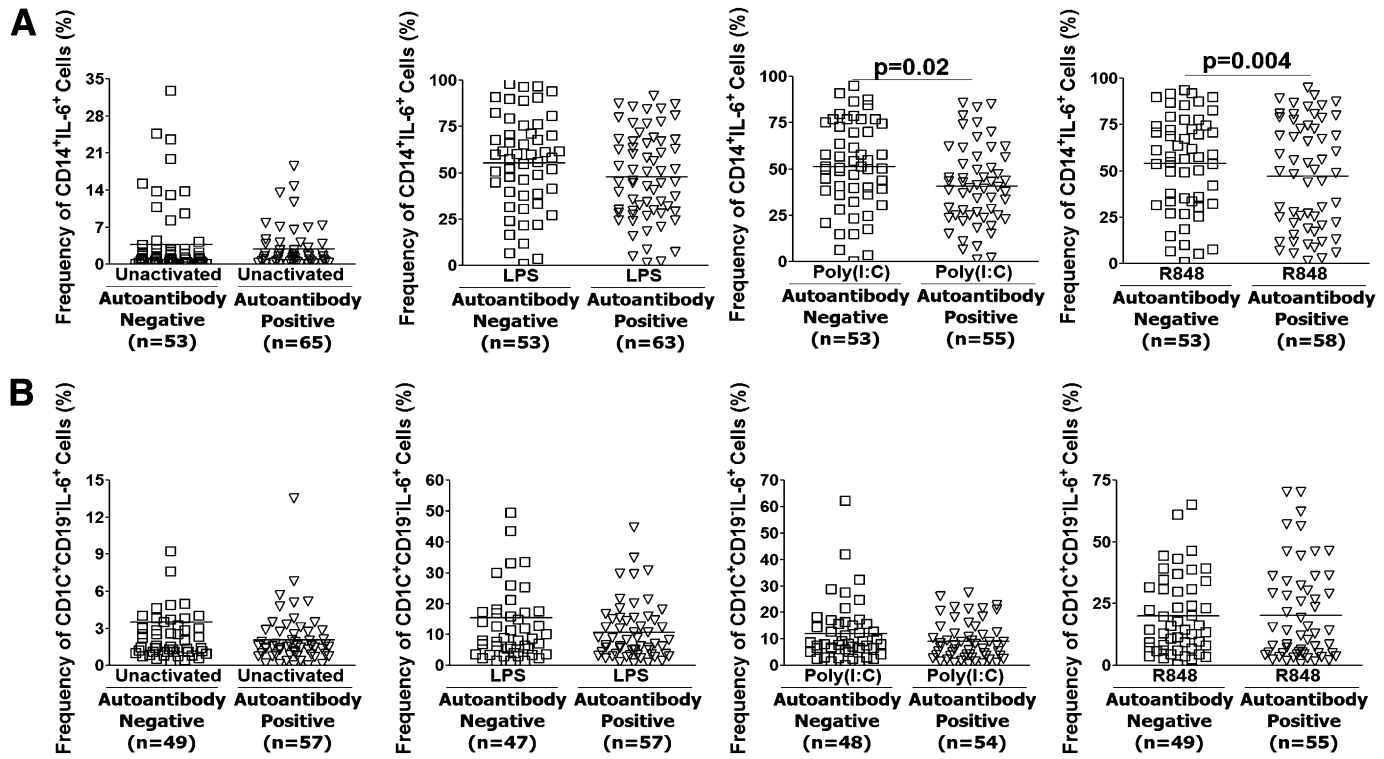

FIG. 4. TLR-induced IL-6 in monocytes and mDCs. PBMNCs were unactivated or stimulated with purified TLR agonists, followed by staining for intracellular IL-6. Each symbol represents an individual percentage of monocytes (*A*) or mDCs (*B*) expressing IL-6 of the total monocytes and mDCs, respectively. The bars indicate the mean values. Significant differences were evaluated using the nonparametric Mann-Whitney *U* test.

Our findings that seropositive subjects have altered IL-1β and IL-6 pathways are compatible with our recent finding that newly diagnosed patients with T1D have higher proportions of ex vivo monocytes secreting IL-1β and elevated numbers of TLR-induced IL-1β−expressing monocytes compared with healthy subjects (11). Our data lend support to the hypothesis that changes in TLR responsiveness are detectable in monocytes and mDCs from humans with ongoing autoimmunity before and after disease onset. In addition, our results are also in accordance with a previous report that patients with T1D have higher frequencies of ex vivo IL-1β–expressing monocytes determined using ELISPOT (21). It was proposed that monocytes could be involved in T1D onset by inducing an IL-17 response (21).

Observations from a number of experimental systems have suggested the potential involvement of the IL-1 pathway in islet destruction. We recently demonstrated that the IL-1 pathway is involved in the proinflammatory response leading to virus-induced T1D (20,22). IL-1, alone or combined with other proinflammatory cytokines such as IFN-β, can cause β-cell destruction in islets from humans and animals and perfused pancreas via pathways involving mitogen-activated protein kinase and nuclear factor (NF)-κB (23–25). Blocking the IL-1 pathway protected from T1D in animal models and treating non–diabetes-prone animals with IL-1 caused transient insulinopenic diabetes (25). Finally, earlier reports have linked IL-1 cytokine family members, including IL-1β, IL-1R1, and IL-1R2, with human T1D (26).

Our finding that subjects at risk for T1D have reduced TLR-induced monocyte expression of IL-6 is reminiscent of our previous observation that PBMNCs from new-onset patients activated with LPS contain lower frequencies of IL-6–expressing mDCs (11). Others have also reported a reduced IL-6 response in monocytes from patients with T1D (27). IL-6 promotes inflammation (28) and adaptive immune responses (29); however, it also suppresses the functions of various cell subsets, including macrophages and synovial fibroblasts. IL-6 induces anti-inflammatory factors, such as IL-1 receptor antagonist and glucocorticoids, inhibits the production of proinflammatory cytokines, such as IL-1, tumor necrosis factor, and IL-12, and downregulates the expression of adhesion molecules (30). Thus, reduced IL-6 responses in seropositive individuals could potentially be part of early mechanisms leading to loss of immune regulation and consequently T1D.

It is currently unclear how dysregulated TLR-induced IL-1β and IL-6 responses in monocytes and mDCs are involved in mechanisms leading to islet destruction. There is also uncertainty regarding the mechanism underlying this alteration. The data demonstrating that subjects with more than one autoantibody have similar TLR-induced IL-1β and IL-6 responses as subjects with only one autoantibody is interesting given the large difference in the risk for T1D between these groups (5). One potential explanation is that the increased TLR responses seen in seropositive subjects may be linked with mechanisms triggering anti-islet autoimmunity rather than progression to T1D. The interpretation of these data should be constrained by the caveat that most of the seropositive subjects included in this study expressed only one or two autoantibodies. It remains to be determined whether monocytes and mDCs from individuals with three autoantibodies react differently to TLR ligation compared with subjects with one and

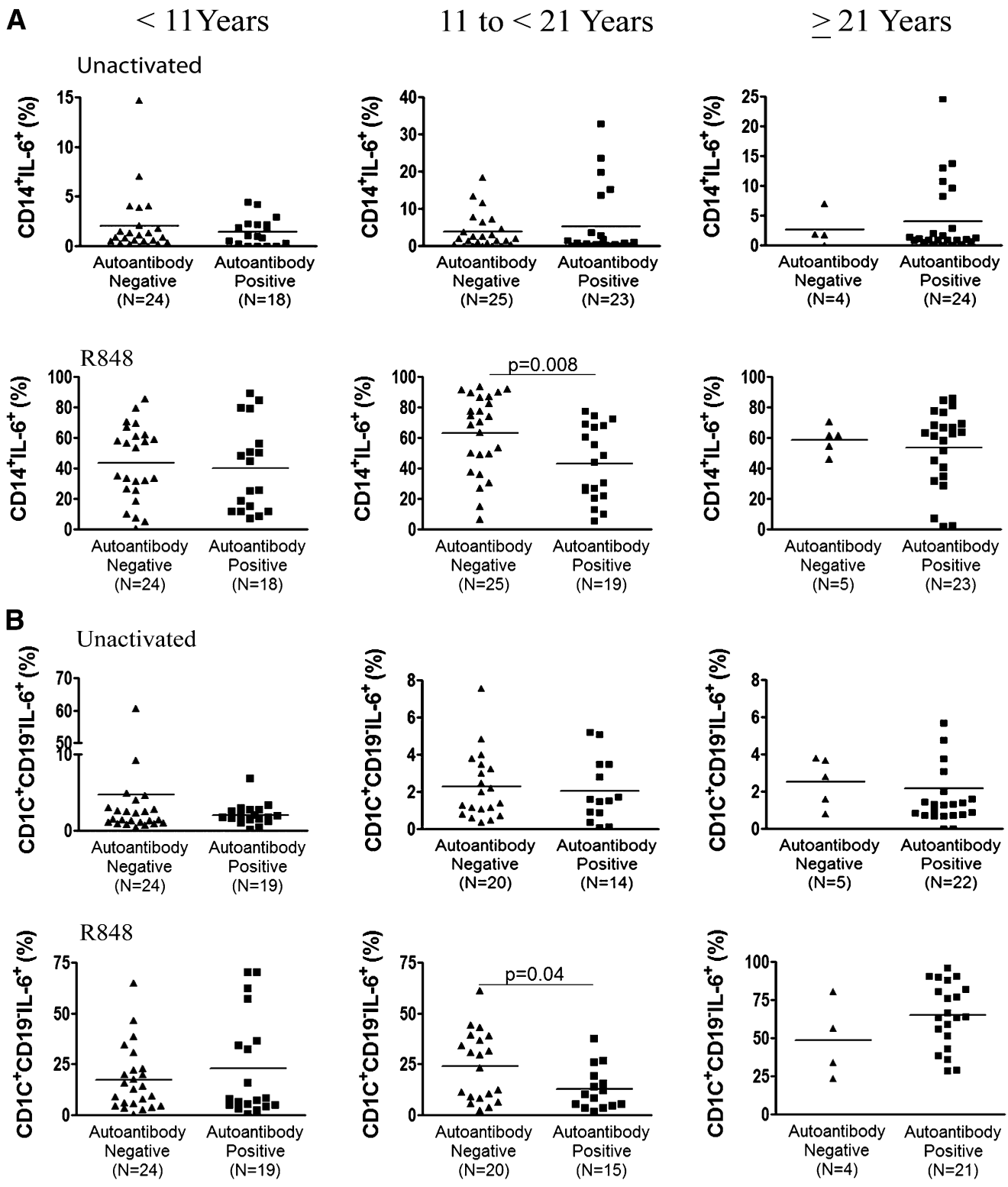

FIG. 5. Correlation between TLR-induced IL-6 in monocytes and mDCs vs. age. IL-6 responses were evaluated in subjects grouped by age: <11 years (young children), from 11 to <21 years (adolescents), and ≥21 years (adults). Each symbol represents an individual frequency of IL-6–expressing monocytes (*upper panel*) and mDCs (*lower panel*) after stimulation with R848. The bars represent the mean values. *P* values were evaluated using the nonparametric Mann-Whitney *U* test.

two autoantibodies. Also intriguing is the observation that altered IL-1β responses are more prevalent in young children (<11 years) compared with IL-6 responses that appeared to be more readily detectable in adolescents. It is tempting to speculate that the altered innate immune pathways detectable in seropositive individuals could be occurring at different ages or disease stages.

The altered ability of monocytes and mDCs to respond to TLR ligation could be involved in disease mechanisms by altering the balance between proinflammatory and

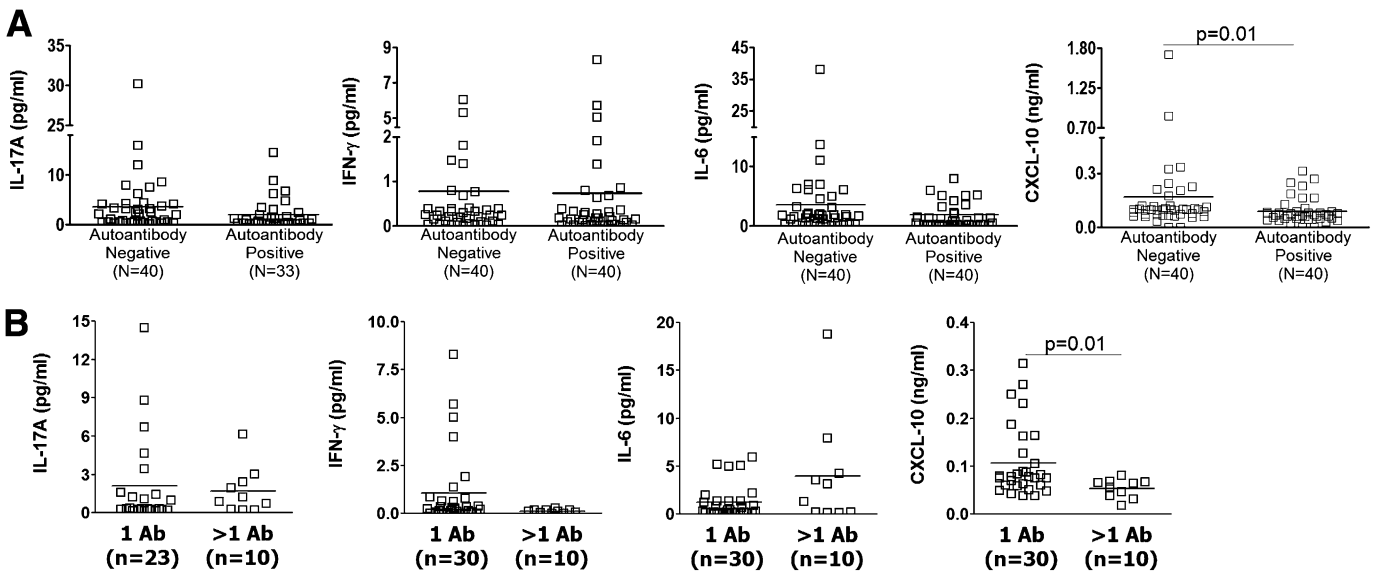

FIG. 6. Serum cytokine and chemokine expression levels and correlation with autoantibody number. *A*: Each symbol represents an individual level of the indicated cytokine or chemokine. *B*: The serum expression level of the indicated cytokine or chemokine was stratified into two groups based on the expression of one and more than one anti-islet autoantibodies. The bars indicate mean values. For statistical comparisons, cytokine concentrations were log-transformed to obtain approximately normally distributed measures and then analyzed using ANOVA.

regulatory mechanisms in the periphery and/or the pancreatic islets. This could result in greater islet inflammation and anti-islets T-cell upregulation (31). Dysregulated TLR-induced cytokine and chemokine responses could be induced, at least in part, by genes linked with mediating innate immune functions or innate regulatory mechanisms. Our data suggest that the mechanism of altered TLR-induced IL-1β and IL-6 responses in seropositive subjects does not involve altered TLR-induced NF-κB phosphorylation or TLR expression (data not shown). It is important to note, however, that although all study participants were free of hyperglycemia, we are unable to exclude the possibility that the altered innate immunity we observed in these subjects is in fact a result of subtle changes in glucose metabolism triggered by ongoing proinflammatory responses in pancreatic islets (32,33).

In contrast to our previously published data documenting elevated levels of IFN-γ, IL-1β, and CXCL-10 in sera from new-onset patients (11), we observed that serum expression levels of CXCL-10 were lower in seropositive compared with seronegative subjects, whereas the amount of IFN-γ was similar in these subject groups. Why seropositive individuals have reduced amounts of CXCL-10 in their blood is unknown. This reduction could be a consequence of recruitment of chemokine-secreting monocytes, DCs, or B lymphocytes from the peripheral blood to inflamed tissues. The observation that the level of serum CXCL-10 is reduced in subjects with more than one versus only one autoantibody is compatible with this hypothesis, as it would be reasonable to hypothesize that individuals with more than one autoantibody may have a higher degree of inflammation, resulting in the recruitment of greater numbers of CXCL-10–producing cells to inflamed islets. The possibility that CXCL-10 is directly involved in the inflammation leading to T1D is supported by a number of reports. CXCL-10 is detectable in human pancreatic islets from patients with new-onset T1D (34) and can also be found in pancreatic lymph nodes and islets from animal models of virus-induced T1D (35; our unpublished data). Our opposing observations indicating an increase versus no change or a decrease in proinflammatory cytokine and chemokine levels in new-onset patients versus prediabetic individuals, respectively (current report; 11), could imply that alterations detected in subjects with new-onset T1D may be at least partly linked with late stages of T1D or hyperglycemia and inflammation previously documented in patients with T1D (32).

In summary, our data demonstrate that individuals with active anti-islet autoimmunity have altered TLR-induced IL-1β– and IL-6–signaling pathways before disease onset. Future longitudinal studies in prediabetic individuals progressing to T1D will be required to evaluate the potential link between the innate immune system and disease onset. Studies to identify early immune pathways involved in T1D are critical not only for designing anti-inflammatory therapeutic interventions but also for identifying biomarkers that could be used for monitoring inflammation and disease progression.

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A.K.A. performed the experiments involving in vitro TLR activation, flow cytometry analyses, serum cytokine measurements, and the Type 1 Diabetes TrialNet Natural History demographic data analysis. M.R. provided the DAISY samples, reviewed the data, and participated in writing the manuscript. F.D. performed statistical analyses. K.W.

handled the DAISY blood samples. P.A.G. provided the Type 1 Diabetes TrialNet Natural History blood samples, reviewed the data, and participated in writing the manuscript. D.Z. reviewed and analyzed the data and wrote the manuscript. D.Z. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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