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A Vitamin D Analog Down-Regulates Proinflammatory Chemokine Production by Pancreatic Islets Inhibiting T Cell Recruitment and Type 1 Diabetes Development[1]

Nadia Giarratana, Giuseppe Penna, Susana Amuchastegui, Roberto Mariani, Kenn C. Daniel, and Luciano Adorini[2]

Type 1 diabetes (T1D) is an autoimmune disease characterized by leukocyte infiltration into the pancreatic islets, and we have previously shown that treatment of adult NOD mice with a vitamin D analog arrests the progression of insulitis, blocks Th1 cell infiltration into the pancreas, and markedly reduces T1D development, suggesting inhibition of chemokine production by islet cells. In this study, we show that all TLRs are expressed by mouse and human islet cells, and their engagement by pathogen-derived ligands markedly enhances proinflammatory chemokine production. The vitamin D analog significantly down-regulates in vitro and in vivo proinflammatory chemokine production by islet cells, inhibiting T cell recruitment into the pancreatic islets and T1D development. The inhibition of islet chemokine production in vivo persists after restimulation with TLR ligands and is associated with up-regulation of IκBα transcription, an inhibitor of NF-κB and with arrest of NF-κBp65 nuclear translocation, highlighting a novel mechanism of action exerted by vitamin D receptor ligands potentially relevant for the treatment of T1D and other autoimmune diseases. ***The Journal of Immunology*****, 2004, 173: 2280–2287.**

The key feature of type 1 diabetes (T1D)[3] is a cell-mediated destruction of the insulin-secreting β cells in the pancreatic islets (1). Transfer experiments in the NOD mouse, which develops a disease resembling the human counterpart (2), have shown that T lymphocytes cause T1D (3). However, the events that initiate the disease and the factors driving the progressive infiltration of leukocytes, in particular T and B lymphocytes, dendritic cells, and macrophages, into the pancreatic islets are still incompletely understood.

Chemokines, the central mediators of cell trafficking, have been implicated in the development of NOD and human T1D (4). Chemokine genes are present within the diabetes susceptibility locus *Idd4* in the NOD mouse (5), one of the ~20 loci associated with T1D development. Several chemokines are produced by pancreatic β cells, including CCL2 (6, 7), CCL5, CXCL9, and CXCL10 (7), suggesting a direct role of β cells in leukocyte recruitment into the pancreatic islets. Chemokines have been implicated as recruiters of pathogenic (8–10) and regulatory (11) T cells to the pancreatic islets, highlighting their role in T1D pathogenesis.

Chemokines are constitutively produced by a wide variety of cell types, and they are up-regulated in response to infectious agents and in inflammatory conditions (12). The host defense against microbial pathogens is triggered by the recognition of conserved motifs in infectious microorganisms mediated by TLRs, surface molecules able to recognize distinct structural components of pathogens (13). Activation of signal transduction pathways by TLRs leads to up-regulation of different genes that operate in host defense, including costimulatory molecules, cytokines, and chemokines (14). Provocation of T1D has been associated with infectious agents (15, 16) but a clear-cut link has not yet been established, and infections can also protect from T1D (17), suggesting that TLR activation is only one of the many factors modulating induction of autoimmune diabetes.

Multiple immunointervention strategies targeting different components of innate and adaptive immune responses have been found effective in inhibiting T1D development in the NOD mouse (18), including calcitriol (19) and other vitamin D analogs (20). Our previous work has shown that treatment of adult NOD mice with the vitamin D analog BXL-219 arrests the progression of insulitis, blocks Th1 cell infiltration into pancreatic islets, and prevents T1D, suggesting that down-regulation of chemokine production by islet cells could represent an important mechanism of action leading to inhibition of T1D development (21).

In the present study, we have found that transcripts encoding all TLRs are expressed by mouse and human islet cells and they are functional, as demonstrated by the marked up-regulation of chemokine production following TLR engagement by specific ligands. This suggests that TLR-mediated up-regulation of proinflammatory chemokine production by islet cells plays an important role in the early events leading to leukocyte infiltration into the pancreatic islets and offers a novel explanation for the association between infectious agents, in particular viruses, and development of T1D. We have verified the hypothesis that arrest of insulitis and block of Th1 cell infiltration into the pancreas by treatment of NOD mice with BXL-219 is associated with reduced chemokine production by islet cells (21) by demonstrating that this agent significantly down-regulates in vitro and in vivo proinflammatory chemokine production by islet cells, inhibiting T cell recruitment into the pancreatic islets and T1D development. This is associated with up-regulation in

BioXell, Milan, Italy

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[2] Address correspondence and reprint requests to Dr. Luciano Adorini, BioXell, Via Olgettina 58, I-20132 Milan, Italy. E-mail address: luciano.adorini@bioxell.com

[3] Abbreviations used in this paper: T1D, type 1 diabetes; VDR, vitamin D receptor; HPRT, hypoxanthine guanine phosphoribosyltransferase; LCMV, lymphocytic choriomeningitis virus.

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islet cells of transcripts encoding IκBα, an inhibitor of the transcription factor NF-κB controlling immune and inflammatory responses (22).

Materials and Methods

Mice

BALB/c mice were purchased from Charles River Breeding Laboratories (Calco, Italy). NOD.SCID, NOD, and NOR mice from The Jackson Laboratory (Bar Harbor, ME) were isolator reared at Charles River Breeding Laboratories. Mice were kept under specific pathogen-free conditions. All animal studies have been approved by the Institutional Review Board.

Vitamin D analog

1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D_3 (BXL-219, formerly Ro 26-2198), was dissolved in ethanol and diluted in culture medium. NOD.SCID mice were dosed orally five times per week with vehicle (miglyol 812) alone or containing BXL-219 (0.1 μg/kg) for 8 wk from 8 wk of age.

Islet isolation and cell culture

Mouse pancreatic islets, purified by density gradient centrifugation following collagenase digestion of the pancreas, were handpicked and cultured in flat-bottom 96-well plates (35 islets/well) in 250 μl of RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal clone serum (HyClone, Logan, UT), 50 μM 2-ME, 2 mM L-glutamine, and 50 μg/ml gentamicin (Sigma-Aldrich). NIT-1 cells, a pancreatic β cell line established from transgenic NOD/Lt mice harboring a hybrid rat insulin-promoter/SV40 large T-Ag gene (23), were obtained from the American Type Culture Collection (Manassas, VA). Handpicked human islets were purified as described previously (24) and cultured as outlined above.

Real-time quantitative RT-PCR

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was performed, and real-time quantitative RT-PCR of total cDNA using specific primers was conducted using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and either TaqMan or SYBRgreen chemistry. The primer sequences used are available upon request. Relative quantification of target cDNA was determined by calculating the difference in cross-threshold (ΔC_T) values after normalization to hypoxanthine guanine phosphoribosyltransferase (HPRT) or GAPDH signals, according to the formula $2^{-\Delta CT}$. To exclude amplification of genomic DNA, RNA samples were treated with DNase (Sigma-Aldrich).

Chemokine quantification

Chemokines were quantified by two-site ELISA. Paired mAbs specific for CXCL10, CCL5, CCL3 (PeproTech, Rocky Hill, NJ), CCL2 (BD Pharmingen, San Diego, CA), and CCL17 and CCL21 (R&D Systems, Minneapolis, MN) were used as described previously (21). Detection limits were 100 pg/ml for CCL2, 15 pg/ml for CXCL10, CCL5, CCL17, and CCL21, and 1 pg/ml for CCL3.

TLR function

Handpicked pancreatic islets were cultured with 10 μg/ml peptidoglycan from *Staphylococcus aureus* (Fluka, Buchs, Switzerland), 30 μg/ml poly(I:C) (Sigma-Aldrich), 1 μg/ml *Salmonella minnesota* LPS (Sigma-Aldrich), 30 ng/ml flagellin (Alexix, Carlsbad, CA), 1 μg/ml imidazoquinoline compound R848 (InvivoGen, San Diego, CA), 1 μM CpG 1826 (InvivoGen), or 5 μg/ml CpG 1668 oligodeoxynucleotide (MWG, Ebersberg, Germany). Endotoxin content in TLR ligand preparations, except LPS, was $<$0.007 endotoxin units/ml, as determined by *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD). After 72 h of culture, supernatants were collected and chemokine concentrations were determined by ELISA.

Immunofluorescence

Immunofluorescent stainings were conducted on 5-μm-thick sections from NOD.SCID pancreas, NIT-1 cells, or from handpicked human islets adhered to poly-L-lysine-coated slides. After five washes with wash buffer (0.45 M NaCl, 0.24 M Na_2HPO_4, 0.24 M NaH_2PO_4, and 0.3% Triton X-100), NIT-1 cells were incubated overnight at 4°C with biotinylated rat anti-mouse TLR4 (eBioscience, San Diego, CA), washed again, and incubated for 90 min with Rhodamine Red-X-streptavidin (Jackson ImmunoResearch, West Grove, PA). NOD.SCID and human islets were incubated overnight at 4°C with affinity-purified goat anti-human TLR3 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) followed by Rhodamine Red-X-conjugated AffiniPure donkey anti-goat IgG (Jackson ImmunoResearch), mouse mAb anti-human TLR4 (HTA125; Santa Cruz Biotechnology) followed by Cy2-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch), or with biotinylated rat anti-mouse TLR4 (eBioscience) followed by Alexa Fluor 488-streptavidin (Molecular Probes, Eugene, OR), or with guinea pig anti-pig insulin (Cytomation-Dako, Carpinteria, CA) followed by Cy5-conjugated AffiniPure donkey anti-guinea pig IgG (Jackson ImmunoResearch). For RelA staining, islets were permeabilized with 0.1% Triton X-100 for 4 min at 4°C and stained with rabbit anti-NF-κBp65 Ab (Santa Cruz Biotechnology), followed by polyclonal anti-rabbit FITC (Sigma-Aldrich). After washing, islets were stained with 2.5 μg/ml propidium iodide. Negative controls were performed by incubation with appropriate isotype-matched primary Abs. The slides were then washed again and mounted with 90% glycerol/PBS. Slides were analyzed with an MRC-1024 confocal microscope (Bio-Rad, Hercules, CA). Images were acquired and processed with Laser Sharp 3.2 software (Bio-Rad).

Cell transfer

Spleen cells from early-diabetic, 16-wk-old NOD mice were incubated with anti-CD4 and anti-CD8 mAb-coated microbeads (Miltenyi Biotec, Auburn, CA) and positively selected using Mini-MACS columns (Miltenyi Biotec). Purified spleen cells were adoptively transferred (10^7/recipient) by i.v. injection into NOD.SCID mice. Pancreatic single-cell suspensions, prepared as described elsewhere (21), were stained with anti-CD45 mAb immediately after isolation.

Results

Chemokine production by islet cells

Overt T1D is preceded by leukocyte infiltration into the pancreatic islets, implying a key role for chemokines produced by islet cells in disease pathogenesis. In the NOD mouse, islets are progressively infiltrated, from ~4 wk of age by lymphocytes, macrophages, and dendritic cells, and to exclude the contribution of infiltrating cells to chemokine production, islets were routinely obtained from immunodeficient NOD.SCID mice. We could show, using real-time RT-PCR, basal ex vivo expression of chemokine transcripts in freshly isolated NOD.SCID islets (Fig. 1). PCR products were amplified and confirmed by gel electrophoresis (Fig. 1*a*), and the relative expression of chemokines normalized to HPRT is shown in Fig. 1*b*. Transcripts encoding CXCL10 were prominently expressed, followed by CCL22, CCL21, CCL3, CCL17, and CCL2. We then examined constitutive chemokine production in NOD.SCID islet cultures by ELISA (Fig. 1*c*). Copious amounts of CXCL10 were secreted in the supernatant during a 72-h culture (1034 pg/ml), as well as appreciable levels of CCL2 (451 pg/ml) and CCL5 (382 pg/ml), and low but detectable levels of CCL3 (5 pg/ml). Levels of CCL17 and CCL21 were below the detection limits. Finally, we compared chemokine production by islet cultures from NOD.SCID, NOR, and BALB/c mice (Fig. 1*d*). NOR and BALB/c islets secreted, compared with NOD.SCID islets, ~50% lower levels of CXCL10, CCL2, and CCL5.

These results demonstrate that pancreatic islets constitutively produce chemokines and notably relatively high levels of CXCL10, CCL2, and CCL5. Islets from the diabetes-resistant NOR mouse, characterized by negligible insulitis (25), as well as from the BALB/c mouse, secrete lower levels of these chemokines compared with NOD.SCID islets, suggesting that constitutive secretion of proinflammatory chemokines by islet cells may represent a contributing factor in the development of T1D.

TLR expression by mouse and human islet cells

T1D is a multifactorial disease, with a combination of genetic and environmental factors contributing to β cell destruction (26). The early events in the pathogenesis of T1D are still poorly understood but infectious agents, in particular viruses, have been implicated in

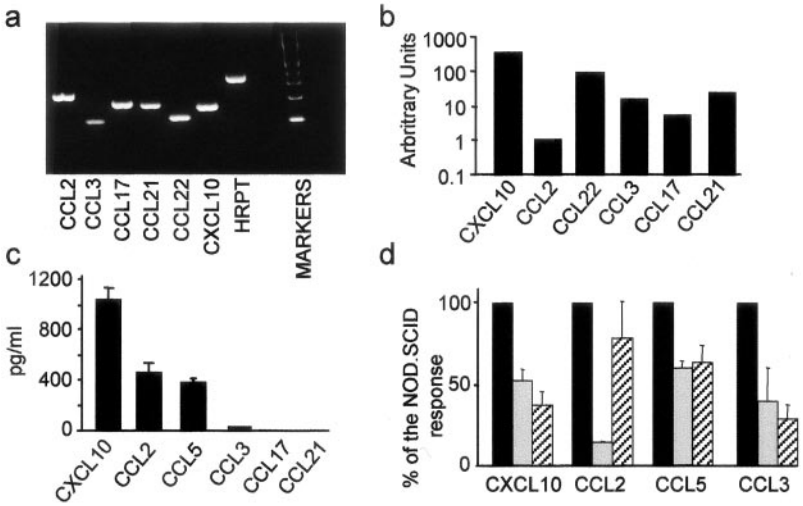

FIGURE 1. Chemokine production by NOD.SCID pancreatic islets. *a*, Gel electrophoresis of *CCL2*, *CCL3*, *CCL17*, *CCL21*, *CCL22*, *CXCL10*, and *HPRT*-amplified products. Total RNA was isolated from handpicked NOD.SCID pancreatic islets. *b*, Quantification of *CXCL10*, *CCL2*, *CCL22*, *CCL3*, *CCL17*, and *CCL21* expression by SYBRgreen chemistry on the same preparation of NOD.SCID islets. The levels of mRNA are shown as arbitrary units normalized to *HPRT* expression. Data are from a representative experiment of three performed. *c*, Constitutive secretion of CXCL10, CCL2, CCL5, CCL3, CCL17, and CCL21 by unstimulated NOD.SCID pancreatic islets. Islets were cultured for 72 h, and the chemokine concentration in the supernatants was measured by ELISA. The data represent the mean ± SE of three to five independent islet culture wells per group from three to eight individual experiments. *d*, Constitutive secretion of CXCL10, CCL2, CCL5, and CCL3 by NOR (▨) and BALB/c (▨) compared with NOD.SCID (■) pancreatic islets. Islets were cultured for 72 h, and the chemokine concentration in the supernatants was measured by ELISA. Values are represented as a percentage of the NOD.SCID pancreatic islet secretion. The data represent the mean ± SE of three to five independent islet culture wells per group from two to three individual experiments.

disease provocation (15). The host defense against microbial pathogens is triggered by the recognition of conserved motifs in infectious microorganisms mediated by TLRs, surface molecules able to recognize distinct structural components of pathogens (13), and activation of signal transduction pathways by TLRs leads to up-regulation of different genes that operate in host defense, including costimulatory molecules, cytokines, and chemokines (14). Using real-time RT-PCR, we have observed expression of mRNA transcripts encoding TLR1 through TLR9 in islet cells from NOD .SCID, NOR, and BALB/c mice (Fig. 2*a*). No major differences in TLR expression among the different mouse strains tested were discernible. In addition, NIT-1 cells, a pancreatic β cell line established from NOD mice (23), expressed all TLR transcripts (data not shown). All TLR transcripts were also expressed by human islet cells (Fig. 2*c*) and confocal microscopic analysis demonstrated a broad expression of TLR3 and TLR4 by NOD.SCID and human pancreatic islet cells, including insulin-producing β cells, as shown by colocalization of insulin and TLR expression (Fig. 2, *b* and *d*). Thus, all TLRs are expressed in mouse and human pancreatic islets, not only by β cells but also by other islet cell types. These results extend recent studies showing expression of TLR2, 3, 4, and 9 by mouse and human pancreatic islet cells (27, 28).

Enhancement of islet chemokine production by stimulation with TLR ligands

To determine whether the TLRs expressed by islet cells were functional, we analyzed chemokine production by NOD.SCID islets in response to TLR ligands. A marked up-regulation of CXCL10, CCL5, CCL2, and CCL3 secretion was observed following islet stimulation with specific TLR ligands, such as peptidoglycan for TLR2, poly(I:C) for TLR3, LPS for TLR4, flagellin for TLR5, R848 for TLR7, and CpG for TLR9 (Fig. 3). A particularly marked enhancement of chemokine production was induced by poly(I:C), but all of the different stimuli tested induced a significantly higher chemokine production compared with basal levels. Poly(I:C) trig-

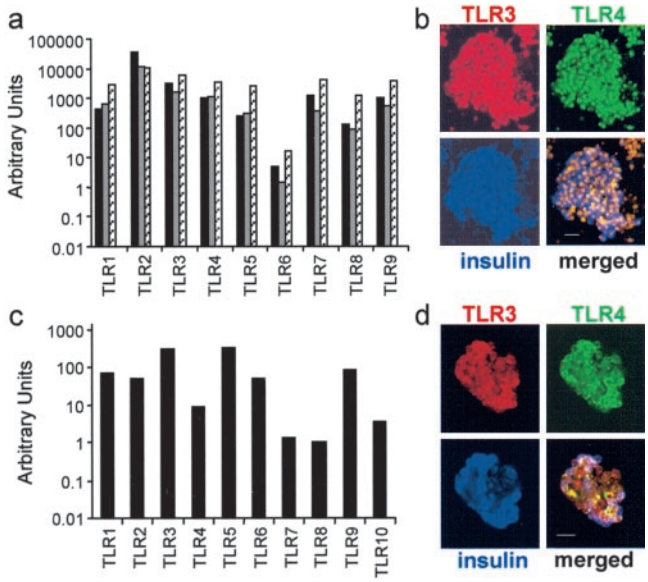

FIGURE 2. TLR expression in pancreatic islets. *a*, Quantification of *TLR* mRNA expression by real-time RT-PCR on freshly isolated NOD.-SCID (■), NOR (▨), or BALB/c (▨) pancreatic islets. The levels of mRNA are shown as arbitrary units normalized to *GAPDH* expression. Data are from one representative experiment of three performed. *b*, Individual and merged stainings for TLR3, TLR4, and insulin in NOD.SCID islet cells. No staining was revealed in slides incubated with isotype-matched primary Ab controls (data not shown). *c*, Quantification of *TLR* mRNA expression by real-time RT-PCR in handpicked human islets. The levels of mRNA are shown as arbitrary units normalized to *GAPDH* expression. *d*, Individual and merged stainings for TLR3, TLR4, and insulin in human islet cells. No staining was revealed in slides incubated with isotype-matched primary Ab controls (data not shown). Bars correspond to 20 μm.

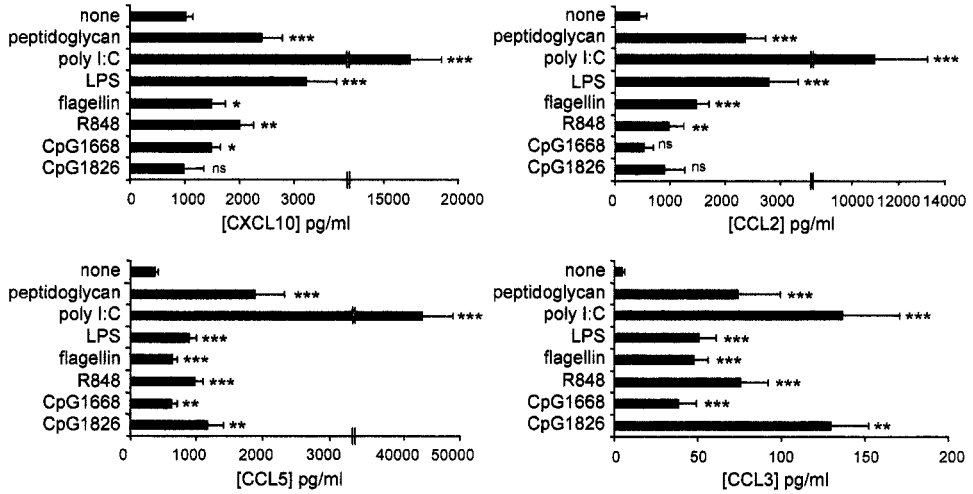

FIGURE 3. Enhancement of CXCL10, CCL2, CCL5, and CCL3 production by NOD.SCID islets stimulated with TLR ligands. NOD.SCID islets were cultured with peptidoglycan, poly(I:C), LPS, flagellin, R848, CpG 1668, or with CpG 1826 for 72 h and chemokine concentration in the supernatants was measured by ELISA. The data represent the mean ± SE of three to five independent islet culture wells per group from three to eight individual experiments. ***, $p < 0.0005$; **, $p < 0.005$; and *, $p < 0.05$ by Mann-Whitney *U* test.

gered TLR3 also in human islet cells, as shown by the increased production of CXCL10 from 219 ± 46 to 6152 ± 398 pg/ml detected by two-site ELISA (mean ±SE from seven individual culture wells). These results demonstrate that TLRs expressed by islet cells are functional and suggest that different microorganisms can enhance chemokine production by islet cells, thereby potentially contributing to T1D pathogenesis.

The vitamin D analog BXL-219 inhibits in vitro chemokine production by islet cells

The vitamin D analog BXL-219 arrests insulitis and blocks Th1 cell infiltration into the pancreatic islets (21), suggesting that its capacity to inhibit T1D may depend on down-regulation of chemokine production by islet cells. To verify this possibility, we first demonstrated expression of the vitamin D receptor (VDR) in islet cells by real-time RT-PCR (data not shown) and then examined the capacity of BXL-219 to inhibit in vitro proinflammatory chemokine production by purified NOD.SCID, NOD, and human islets. BXL-219 significantly down-regulated mRNA expression and constitutive production of CXCL10 by NOD.SCID islets (Fig. 4*a*). CCL2 and CCL5 production was also significantly reduced, with a decrease of ~25–50% (Fig. 4*b*). Although in this study we have routinely analyzed NOD.SCID rather than NOD islets to avoid the confounding factor represented by islet leukocyte infiltration, it was of interest to examine the capacity of BXL-219 to inhibit mRNA expression and constitutive production of CXCL10 by NOD islet cells. Data in Fig. 4*c* demonstrate a higher constitutive production of CXCL10 by NOD compared with NOD.SCID islets, probably due to the contribution of infiltrating leukocytes. In NOD islets, addition of BXL-219 inhibited by ~50% CXCL10 transcript expression and by >60% the constitutive secretion of CXCL10 (Fig. 4*c*). A marked effect was also exerted by BXL-219 on human islets, with a reduction in CXCL10 production of ~70% (Fig. 4*d*). Microscopic examination did not reveal any obvious effect of BXL-219 on islet cell viability. To determine whether BXL-219 could modulate TLR expression, islets were incubated with or without BXL-219 for 2 h and TLR transcripts were analyzed by real-time RT-PCR. Results in Fig. 4*e* show negligible effects of BXL-219 on TLR expression, whereas the mRNA encoding 25-hydroxyvitamin D-24-hydroxylase (*CYP24*), a primary vitamin D-responsive gene (29), is up-regulated by ~100-fold, demonstrating the capacity of NOD islet cells to respond to VDR ligands. Similar results were obtained with NOD.SCID islets (data not shown). These data indicate that BXL-219 can down-regulate in vitro the

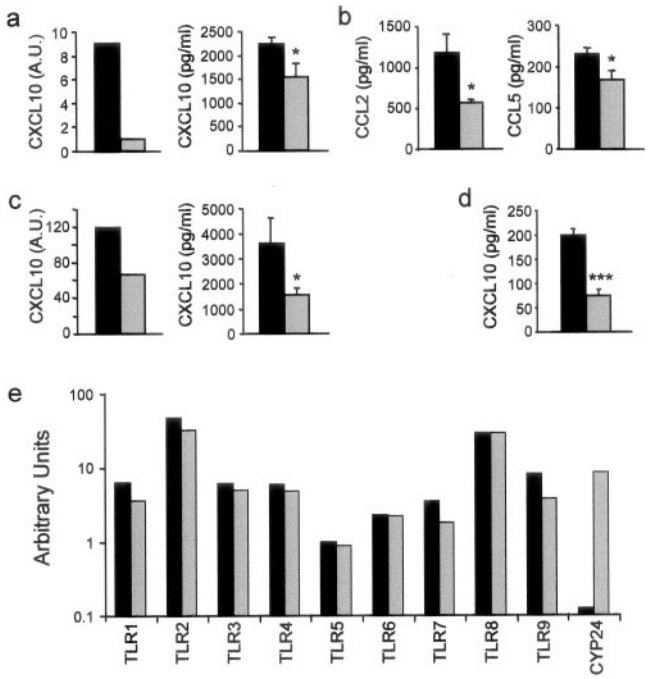

FIGURE 4. BXL-219 down-regulates chemokine production in vitro by mouse and human islets. *CXCL10* mRNA expression detected by real-time RT-PCR using TaqMan chemistry, shown as arbitrary units (A.U.) normalized to *GAPDH* expression, was measured in NOD.SCID and NOD islets (*a* and *c*, respectively, *left panels*) cultured for 2 h with 100 nM BXL-219 (▨) or without (■). NOD.SCID (*right panel*, *b*), NOD (*c*), or human (*d*) islets were cultured for 72 h with 100 nM BXL-219 (▨) or without (■). The concentration of the indicated chemokines was measured in culture supernatants by two-site ELISA. Bars represent mean ±SE from three independent experiments. *, $p < 0.05$ and ***, $p < 0.001$ by one-tailed Student's *t* test. *e* shows, as arbitrary units normalized to *GAPDH* expression, *TLR*, and *CYP24* mRNA levels detected by real-time RT-PCR using TaqMan chemistry in NOD islets cultured for 2 h with 100 nM BXL-219 (▨) or without (■).

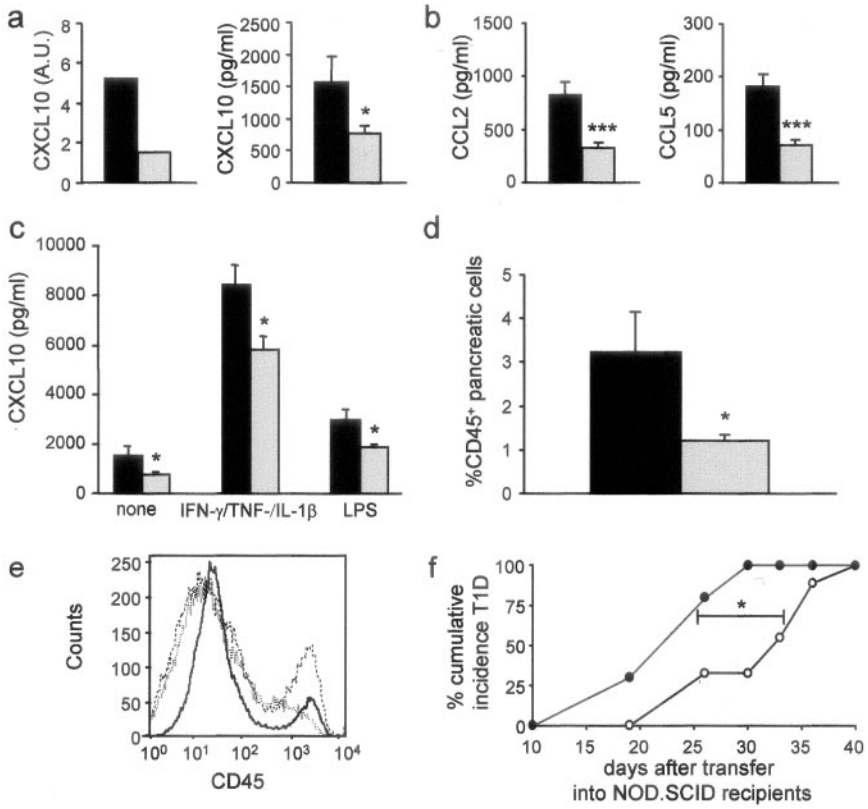

FIGURE 5. BXL-219 treatment down-regulates islet chemokine production in vivo, reduces T cell recruitment to the pancreas, and delays T1D development. Eight-week-old NOD.SCID mice were treated with vehicle (■) or with BXL-219 (▨). Bars represent mean ±SE from individual mice. *, $p < 0.05$ and ***, $p < 0.001$ by one-tailed Student's *t* test. *a*, Constitutive *CXCL10* mRNA expression (expressed as arbitrary units, A.U.) and protein production by islets purified from vehicle ($n = 8$) or BXL-219-treated ($n = 7$) NOD.SCID mice. *b*, Constitutive CCL2 and CCL5 secretion by islets purified from vehicle ($n = 7$) or BXL-219-treated ($n = 8$) NOD.SCID mice. *c*, CXCL10 secretion by islets purified from vehicle ($n = 7$) or BXL-219-treated ($n = 8$) NOD.SCID mice, unstimulated or stimulated in vitro with a mixture of proinflammatory cytokines (IFN-γ, TNF-α, IL-1β) or with LPS. *d*, At the end of the treatment, NOD.SCID mice were injected i.v. with 10^7 positively selected $CD4^+$ and $CD8^+$ T splenocytes from early-diabetic NOD mice. No further treatment was given to spleen cell recipients. Fourteen days after cell transfer, pancreatic cells isolated from vehicle ($n = 5$) and BXL-219-treated ($n = 5$) NOD.SCID mice were stained with anti-CD45 mAb and analyzed by cytofluorimetry. *e*, Recovery of $CD45^+$ cells from the pancreas of a representative NOD.SCID mouse not receiving cell transfer (dotted line) or transferred with 10^7 positively selected $CD4^+$ and $CD8^+$ T splenocytes from early-diabetic NOD mice following treatment of the recipient with vehicle (broken line) or BXL-219 (solid line). *f*, Cumulative T1D incidence in vehicle ($n = 10$) and BXL-219-treated ($n = 9$) NOD.SCID mice transferred with 10^7 positively selected $CD4^+$ and $CD8^+$ T splenocytes from early-diabetic NOD mice.

constitutive production of proinflammatory chemokines by mouse and human islet cells without affecting TLR expression.

Treatment with BXL-219 down-regulates in vivo chemokine production by islet cells, reduces T cell recruitment to the pancreas, and inhibits T1D development

Next, we examined the capacity of BXL-219 to inhibit chemokine production by islet cells in vivo. NOD.SCID mice were treated five times per week for 8 wk, from 8 to 16 wk of age, with 0.1 μg/kg BXL-219 orally, a protocol known to arrest insulitis and to inhibit T1D development in NOD mice (21). Purified islets from BXL-219-treated NOD.SCID mice expressed a 3-fold lower level of mRNA encoding CXCL10 and produced 50% lower levels of the chemokine (Fig. 5*a*). CCL2 and CCL5 production was decreased by ~70% (Fig. 5*b*). The decreased islet production of CXCL10 induced by BXL-219 treatment persisted following ex vivo islet stimulation with a mixture of proinflammatory cytokines or with LPS, the ligand for TLR4 (Fig. 5*c*). To determine whether down-regulation of chemokine production by treatment with BXL-219 was associated with reduced T cell recruitment to the pancreas and T1D development, NOD.SCID recipients were treated with BXL-219 for 8 wk and transferred, at the end of the treatment, with $CD4^+$ and $CD8^+$ T cells from recently diabetic NOD mice. The down-regulation of chemokine production by islets from BXL-219-treated NOD.SCID mice was associated with a significantly reduced recruitment of T cells from recently diabetic NOD mice into the pancreas of BXL-219-treated NOD.SCID recipients (Fig. 5, *d* and *e*) and with a significant delay in T1D development (Fig. 5*f*).

BXL-219 enhances IκBα transcript expression in islet cells and traps NF-κBp65 into the cytoplasm

NF-κB activation up-regulates in pancreatic β cells expression of genes encoding several chemokines, including CXCL10 and CCL2 (30). NF-κB family proteins, c-Rel, RelA (p65), RelB, NF-κB1 (p50), and NF-κB2 (p52) are present in the cytoplasm in an inactive form as a result of their association with the inhibitor proteins IκBα, IκBβ, and IκBε (22). Since 1α,25-dihydroxyvitamin D_3 has been reported to inhibit the expression of NF-κB1 and c-Rel (31), as well as RelB (32), and to down-regulate NF-κB activation (33), we examined transcripts encoding NF-κB family members in NOD.SCID islets cultured for 18 h with or without BXL-219. No difference was observed in mRNA levels, except for the up-regulation of transcripts encoding *IκBα* and *CYP24*, a primary vitamin D-responsive gene (29) (Fig. 6*a*). To test whether the increased expression of *IκBα* transcripts was associated with a reduced translocation of NF-κB proteins to the nucleus, NOD.SCID islets

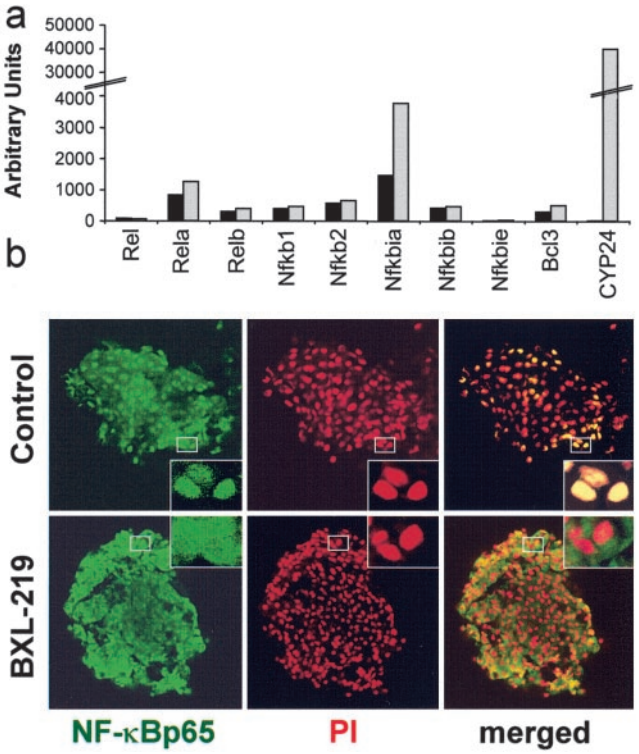

FIGURE 6. BXL-219 enhances *IκBα* transcript expression in islet cells and traps NF-κBp65 into the cytoplasm. *a*, Islets from NOD.SCID mice were cultured for 18 h with (▨) or without (■) 100 nM BXL-219. mRNA levels were detected by real-time RT-PCR using TaqMan chemistry. *b*, After an 18-h culture, islets were incubated with 1 μg/ml LPS for 45 min, fixed, and stained with anti-NF-κBp65 (p65) Ab and propidium iodide (PI). Individual and merged stainings are shown. Original magnifications, ×63; *insets*, ×1000.

cultured for 18 h with or without BXL-219 and stimulated with LPS for 45 min were stained with anti-RelA Ab. Confocal microscopic analysis clearly shows translocation of RelA to the nucleus in control islets, whereas this is retained in the cytoplasm in islets treated with BXL-219 (Fig. 6*b*).

Discussion

In the present study, we show that mouse and human islet cells, including insulin-producing β cells, express all of the TLRs and their triggering markedly increases the secretion of proinflammatory chemokines. These findings suggest that, among the initial events in T1D development, triggering of islet TLRs by microbial products could up-regulate the secretion of chemokines able to attract Th1 cells, macrophages, and dendritic cells. Since these cell types are involved in the pathogenesis of T1D, the TLR-mediated up-regulation of proinflammatory chemokine production by islet cells could represent an important element in the early steps of T1D development leading to leukocyte infiltration into the pancreatic islets.

Our data show that chemokine production by NOD islet cells is targeted by treatment with the vitamin D analog BXL-219, leading to a reduced T cell migration to the pancreas and to delayed T1D development. These novel observations expand the known mechanisms of action exerted by vitamin D analogs in the treatment of T1D and other autoimmune diseases that include arrest of DC maturation, inhibition of T cell responsiveness, and enhancement of regulatory T cells (34–36). The chemokines produced by islet cells are, among those tested, of proinflammatory type: CXCL10, CCL2, CCL5, and low levels of CCL3. No production of the homeostatic chemokines CCL17 and CCL19, potentially able to recruit regulatory/suppressor T cells (11, 37), could be detected, suggesting that their entry into the target organ may occur later in the disease process and depend on chemokine production by other cell types (38). The constitutive and inducible production by islet cells of CXCL10, a ligand for CXCR3 expressed by Th1 cells (12), was most prominent. CXCL10 has previously been shown to be produced by the NOD β cell line NIT-1 stimulated with inflammatory cytokines (7), and the higher constitutive levels we have observed in the NOD background, compared with diabetes-resistant strains, further supports its important role in the pathogenesis of T1D. Our results also show constitutive and inducible production of CXCL10 by human islet cells. CXCL10 has been implicated in human T1D, as elevated serum levels have been observed in diabetes patients and in autoantibody-positive subjects at risk of developing the disease (39, 40). In addition, our results show that CCL5, the ligand for CCR5, another chemokine receptor expressed by Th1 cells (12), is also constitutively produced by islet cells and is markedly up-regulated following TLR ligation. Mouse islet cells produce, besides CCL5, the CCL2 and CCL3 ligands able to recruit $CCR1^+$ and $CCR2^+$ macrophages (12). CCL2 has been shown to be produced also by human islet cells, and it appears to play an important role in the clinical outcome of islet transplantation in T1D patients (24). Islet-produced CXCL10, CCL5, CCL2, and CCL3 could also recruit immature dendritic cells (12). Thus, pancreatic β cells, as well as other islet cell types, produce chemokines potentially able to attract the pathogenic cells ultimately responsible for β cell death.

Production of all chemokines tested was significantly decreased by treatment of mouse and human islets with BXL-219 and administration of BXL-219 to NOD.SCID recipients inhibited the migration of transferred NOD T cells to the pancreas, leading to a significant delay in T1D development. Thus, arrest of insulitis and block of Th1 cell infiltration into NOD pancreatic islets by BXL-219 treatment (21) is associated with inhibition of islet chemokine production, in particular CXCL10, consistent with the decreased recruitment of Th1 cells into sites of inflammation by treatment with an anti-CXCR3 Ab (41) and with the inhibition of acute allograft rejection (42) and the substantial delay of T1D development (7) observed in CXCR3-deficient mice.

The highest levels of chemokine production by islet cells is induced, among all of the different TLR ligands tested, by the viral RNA mimic poly(I:C), a ligand for TLR3 which recognizes extracellular dsRNA released from virus-infected cells (43). A viral component triggering the development of T1D has long been suspected (44), but viruses can also protect from T1D (17, 45). Controversial results have been reported on T1D modulation by poly(I:C): low doses have been shown to protect whereas high doses precipitate T1D development in the diabetes-prone Bio Breeding rat (46, 47). In the NOD mouse, administration of poly(I:C) has been shown to be protective (48) and it failed to enhance T1D development in a colony of BDC2.5-transgenic mice on NOD background exhibiting a low incidence of diabetes (49).

Different mechanisms have been proposed to account for virus-induced autoimmune diabetes, including molecular mimicry, bystander activation of autoreactive T cells, and direct cytopathic effects in virus-infected target cells (15, 50, 51). Intriguingly, infection with lymphocytic choriomeningitis virus (LCMV) in NOD or in LCMV-rat insulin promoter-transgenic mice can abrogate T1D development, and this is associated with LCMV-induced expression of CXCL10 in draining pancreatic lymph node cells, creating a chemokine gradient correlated with the rapid egress of

pancreas-infiltrating lymphocytes (52). In addition to these possible mechanisms, our data may be interpreted to infer that the association between virus infection and T1D could reflect the triggering by viral products of signal transduction via TLRs expressed by islet cells, in particular TLR3 and TLR9, leading to production of proinflammatory chemokines by islet cells that contribute to create the conditions for an autoimmune attack. This pathogenetic mechanism in concert with genetic susceptibility and induction of adaptive immune responses may precipitate autoimmune diseases, as recently shown in the induction of autoimmune myocarditis by a combination of TLR stimulation and CD40-mediated triggering of self-peptide-loaded dendritic cells (53). Consistent with these findings, T1D can be precipitated by a combined treatment with poly(I:C) and insulin B9-23 peptide (54), possibly via poly(I:C)-induced triggering of TLR3 (28). Activation of TLRs in target tissues could be involved also in the pathogenesis of systemic autoimmune diseases, as suggested for TLR9 in systemic lupus erythematosus (55, 56).

Our data showing up-regulation of transcripts encoding IκBα and inhibition of RelA nuclear translocation by BXL-219 demonstrate a novel mechanism of action targeting NF-κB by VDR ligands, in addition to the inhibition of NF-κB1 and c-Rel (31) as well as RelB (32) expression. Interestingly, this mechanism of action has been previously demonstrated for glucocorticoids, anti-inflammatory drugs that bind to a nuclear receptor in the same superfamily as the VDR, by showing that dexamethasone up-regulates the transcription of *Nfkbia*, which results in an increased rate of IκBα synthesis and in reduced NF-κB translocation to the nucleus (57, 58). The up-regulation of transcripts encoding IκBα and the inhibition of RelA translocation to the nucleus by BXL-219 prevent activation of NF-κB, a transcription factor critical for the inflammatory response (22) that also regulates chemokine production by pancreatic β cells (30). The promoter of the *Nfkbia* gene encoding IκBα contains, as the *Relb* gene (32), several vitamin D-responsive elements, some of which are highly conserved between human and mouse homologues (C. Carlberg, personal communication), suggesting a direct transcriptional regulation of IκBα by BXL-219. The targeting by BXL-219 of NF-κB could contribute to explaining the inhibition of proinflammatory chemokine production by islet cells and could open new avenues in the use of VDR ligands as anti-inflammatory agents.

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