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ORIGINAL ARTICLE

Comparison between VDR analogs and current immunosuppressive drugs in relation to CXCL10 secretion by human renal tubular cells

Costanza Sagrinati,[1]* Mariangela Sottili,[1]* Benedetta Mazzinghi,[1] Elisa Borgogni,[1] Luciano Adorini,[2] Mario Serio,[1] Paola Romagnani[1] and Clara Crescioli[1,3]

1 Excellence Center for Research, Transfer and High Education (DENOthe), University of Florence, Florence, Italy
2 Intercept Pharma, Perugia, Italy
3 Department of Health Sciences, Unit of Endocrinology, University of Rome 'Foro Italico', Rome, Italy

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Correspondence
Dr Clara Crescioli, Excellence Center for Research, Transfer and High Education (DENOthe), University of Florence, Viale Pieraccini, 6 – 50139 Florence; and Department of Health Sciences, Unit of Endocrinology, University of Rome 'Foro Italico', Piazza Lauro de Bosis 15 – 00194 Rome, Italy. Tel.:+39 055 4271362; fax: +39 055 4271371; e-mail: c.crescioli@dfc.unifi.it

*These two authors equally contributed to the work.

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Summary

During kidney allograft rejection, CXC chemokine ligand 10 (CXCL10)–CXC chemokine receptor 3 (CXCR3) trafficking between peripheral blood and tissues initiates alloresponse and perpetuates a self-inflammatory loop; thus, CXCL10–CXCR3 axis could represent a pharmacologic target. In this perspective, immunosuppressors targeting graft-resident cells, beside immune cells, could be very advantageous. Vitamin D receptor (VDR) agonists exhibit considerable immunomodulatory properties. This study aimed to investigate whether elocalcitol and BXL-01-0029 could decrease the expression of CXCL10 in activated renal tubular cells *in vitro* and thus be useful in kidney allograft rejection treatment. Experiments were performed in human tubular renal cells stimulated with interferon-γ + tumor necrosis factor-α with and without VDR agonists, tacrolimus, sirolimus, hydrocortisone, methylprednisolone, cyclosporin A and mycophenolate mofetil. CXCL10 protein secretion and gene expression were measured by ELISA and by quantitative PCR. Specific inhibitors were used to investigate intracellular pathways involved in tubular cells activation. For IC_{50} determination and comparison, dose-response curves with VDR agonists, tacrolimus and mycophenolic acid were performed. Elocalcitol and BXL-01-0029 inhibited CXCL10 secretion by renal cells, without affecting cell viability, while almost all the immunosuppressors were found to be ineffective, except for tacrolimus and mycophenolate mofetil. BXL-01-0029 was the most potent drug and, notably, it was found to be capable of allowing reduction in tacrolimus-inhibitory doses. Our data suggest that BXL-01-0029 could potentially be a dose-reducing agent for conventional immunosuppressors in kidney rejection management.

Introduction

Allograft rejection (AR) remains a major concern after kidney transplant, despite the continuous improvements in the immunosuppressive regimens. T-helper 1 (Th1) cell infiltration in kidney allografts considerably contributes to organ damage. Tubular injury is characterized by the presence of CXC chemokine receptor 3 (CXCR3)-bearing T cells and CXCR3-binding chemokines, such as Mig/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11 [1,2]. Th1 cells release proinflammatory cytokines, i.e. interferon (IFN)-γ and tumor necrosis factor (TNF)-α [1], which potently enhance local production of Th1-type chemokines, particularly by tubular epithelial cells [2]. In turn, chemokine deposit at the site of injury further supports leukocyte infiltration and enhances tubular damage

progression [1,2]. CXCL10–CXCR3 trafficking seems to particularly sustain this vicious circle. Indeed, CXCL10 not only mediates leukocyte recruitment but, most likely, initiates the immune response to the antigens [3] during AR. Indeed, CXCL10-blocking reduced Th1-cell recruitment and improved renal function in animals with renal microvascular injury [4]. Notably, CXCL10 intragraft expression correlates with renal AR [5] and high pretransplant CXCL10 serum level in kidney transplant recipients is a predictive biomarker of both rejection episodes and graft loss [6,7]. The critical role of CXCL10 in Th1 type-mediated renal diseases potentially offers also the opportunity for therapeutic intervention.

Conventional immunosuppressive drugs that are used to prevent AR have been designed to specifically target the immune cells. Undoubtedly, interfering with the chemokine network recruiting leukocytes may represent a novel tool to aid AR management in kidney transplantation.

Vitamin D receptor (VDR) agonists are very interesting as potential dose-reducing agents for conventional immunosuppressors in Th1-mediated diseases [8,9], as they themselves behave as immunoregulators in several models of AR [10], inhibiting both acute and chronic graft rejection [11]. Furthermore, VDR activation has beneficial effects in chronic kidney disease patients [12–17] and the renal protective effects of a VDR activator, either alone or in combination therapy, have been recently reported [18,19]. Previous *in vitro* studies on AR in heart transplantation and thyroid autoimmune disease showed that elocalcitol and BXL-01-0029, both VDR agonists retaining vitamin D biologic activity but less hypercalcemic, attenuated Th1-mediated inflammatory responses by targeting CXCL10 secretion in resident and immune cells [20,21].

Elocalcitol has been proposed for benign prostate hyperplasia treatment [22,23] and its anti-inflammatory properties have been documented in experimental model of autoimmune prostatitis [23]; BXL-01-0029, a prodrug of BXL-2198 (also known as BXL-219 and Ro 26-2198), has been shown to be active onto Th1-mediated processes in animal models of type 1 diabetes [24,25] and colonic carcinogenesis [26].

Herein, we aimed to verify whether VDR agonists in terms of their ability to interfere with CXCL10 expression in renal tubular cells could be proposed as immunomodulatory agents in kidney transplant management.

We investigated the effect of elocalcitol and BXL-01-0029 on CXCL10 protein secretion induced by IFN-γ and TNF-α in isolated human proximal tubule epithelial cells, in comparison with immunosuppressors currently used in allograft transplant, such as tacrolimus (FK-506), sirolimus (Sir), hydrocortisone (H), methylprednisolone (MeP), cyclosporin A (CsA) and mycophenolate mofetil (MMF) [27].

Materials and methods

Chemicals

Renal Epithelial Cell Growth Medium (REGM™) was procured from Lonza Group Ltd. (Basel, Switzerland). RPMI 1640, phosphate-buffered saline Ca2+/Mg2+-free (PBS), bovine serum albumin (BSA) fraction V, antibiotics, NaOH, absolute ethanol, EDTA-trypsin solution, Bradford reagent, Ficoll–Hypaque, Phorbol 12-myristate 13-acetate (P), Ionomycin (I), p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, C-Jun NH2-terminal kinase (JNK) inhibitor SP600125, extracellular signal-regulated kinase (ERK) inhibitor U0126, Signal transducer and activator of transcription-1 (Stat1) inhibitor fludarabine, tacrolimus (FK-506), mycophenolic acid (MPA, mycophenolate mofetil active agent), sirolimus (Sir), hydrocortisone (H), methylprednisolone (MeP), cyclosporin A (CsA) were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Nuclear factor-kB (NF-kB) inhibitor BAY 11-7082 was from Vinci Biochem S.r.l. (Vinci, Italy); elocalcitol, BXL-01-0029 were from Bioxell (Milan, Italy).

Interferon-γ, TNF-α, ELISA kit for CXCL10 measurement were from R&D Systems, (Minneapolis, MN, USA). Fetal calf serum (FCS) was from Hyclone (Logan, UT, USA). L-Glutamine, nonessential amino acids, pyruvate, and 2-mercaptoethanol were from Gibco Laboratories (Grand Island, NY, USA). RNeasy Mini reagent kit was from Quiagen Italy (Milan, Italy). TaqMan Reverse Transcription Reagents kit, primer/probe mixes (Taqman Gene® Expression Assays, Applied Biosystem), CXCL10 (ID number Hs00171042-ml), 1× Universal Master Mix were from Applied Biosystems (Forster City, CA, USA). Quantitative PCR human reference total RNA was from Stratagene (La Jolla, CA, USA). Trypan blue was from Euroclone (Pavia, Italy). Plasticware for cell cultures, disposable filtration units for growth media preparation were from Corning (Milan, Italy).

RPTEC cultures

Clonetics® primary human proximal tubule epithelial cells (RPTEC) were purchased from Lonza Group Ltd. and maintained in culture in REGM™ in a fully humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Preparation and isolation of $CD4^+$ T lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy adult anonymous

donors, obtained after the Local Ethical Committee approval, by centrifugation of heparinized blood on Ficoll–Hypaque gradient. Briefly, $CD4^+$ T cells were selected from PBMCs by immunomagnetic cell sorting, using the CD4 isolation kit (Miltenyi Biotec, Bisley, Germany), as detailed elsewhere [28] and maintained in RPMI 1640 supplemented with 2 mmol/l L-glutamine, 1% nonessential amino acids, 1% pyruvate, 2×10^{-5} M 2-mercaptoethanol, 10% FCS. Sorted population purity was >95%.

CXCL10 secretion assay in RPTEC

Four thousand cells per well, seeded in REGM, were processed as described elsewhere [29]. Cells in serum-free medium containing 0.1% BSA and vehicle (absolute ethanol, 0.47%, vol/vol) were used as control

For experiments with inhibitors: cells were stimulated for 24 h with IFN-γ (1000 U/ml) + TNF-α (10 ng/ml), with or without 1-h preincubation with SB203580 (5 μM), LY294002 (15 μM), SP600125 (100 μM), U0126 (20 μM), fludarabine (50 μM) or BAY 11–7082 (20 μM).

For experiments with drugs: cells were stimulated for 24 h with IFN-γ (1000 U/ml) + TNF-α (10 ng/ml), with or without a fixed dose of FK-506 (2.5×10^{-8} M), MPA (8.1×10^{-5} M), Sir (1.6×10^{-8} M), H (2.8×10^{-7} M), MeP (6.7×10^{-7} M), CsA (2.1×10^{-7} M), elocalcitol (10^{-8} M), BXL-01-0029 (10^{-8} M) – the drug concentrations were selected on the basis of their near-therapeutic doses, according to their pharmacokinetics (C_{max} and area under the time–concentration curve, AUC), as previously reported [30] – or a combination of BXL-01-0029 (10^{-9}, 10^{-8} M) and FK-506 (2.5×10^{-9}, 1.2×10^{-8}, 2.5×10^{-8} M). FK-506 (2.5×10^{-9}, 6.2×10^{-9}, 1.2×10^{-8}, 2.5×10^{-8}, 6.2×10^{-8}, 2.5×10^{-7}, 6.2×10^{-7}, 1.2×10^{-6} M), MPA (8.1×10^{-7}, 8.1×10^{-6}, 2×10^{-5}, 4.1×10^{-5}, 8.1×10^{-5}, 1.6×10^{-4}, 3.2×10^{-4}, 8.1×10^{-4} M), elocalcitol (10^{-14}–10^{-6} M) and BXL-01-0029 (10^{-14}–10^{-6} M) were used in dose–response curves.

The supernatant was harvested and kept frozen at −20 °C until the performance of ELISA assays. Experiments were performed in triplicate for three to six times.

ELISA assays

The CXC chemokine ligand 10 (CXCL10) level was measured in cell culture supernatants using commercially available kits (R&D Systems), according to manufacturer's recommendations. The sensitivity and the intra- and interassay coefficients of variation for supernatants were indicated in manufacturer's instructions. Samples were assayed in triplicate. Quality control pools of low, normal, or high concentrations for all parameters were included in each assay. Cytokine secreted amount was measured as pg/μg total protein amount; final results were expressed as percentage of IFN-γ + TNF-α-induced secretion. Protein extraction and measurement to normalize RPTEC secretion were performed as reported elsewhere [29].

RNA extraction and real-time PCR

A quantity of 100 000 cells/well were plated in REGM in 12-well plates and processed as reported elsewhere [29]. RPTEC were stimulated for 24 h with IFN-γ (1000 U/ml) + TNF-α (10 ng/ml), with or without FK-506 (2.5×10^{-8} M), MPA (8.1×10^{-5} M), elocalcitol (10^{-8} M) or BXL-01-0029 (10^{-8} M). Cells in serum-free medium containing 0.1% BSA and vehicle (absolute ethanol, 0.47%, vol/vol) were used as control. Total RNA from the cells was extracted with the RNeasy Mini reagent kit according to manufacturer's recommendations. RNA concentration and quality were measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). TaqMan RT-PCR was performed as described elsewhere [29]. Quantization of CXCL10 was performed using Taqman Gene® Expression Assays. The amount of target was given by $2^{-\Delta\Delta C_t}$ calculation, as previously reported [29]. Experiments were performed three times; results were expressed as fold increase versus IFN-γ + TNF-α-induced expression, taken as 1.

Cell viability

For cell viability assays: (1) RPTEC (30 000 cells/well) were seeded in 12-well plates, maintained in serum-free medium for 24 h and then incubated with FK-506 (2.5×10^{-8} M), MPA (8.1×10^{-5} M), elocalcitol (10^{-8} M) or BXL-01-0029 (10^{-8} M) for 1–6 days. Cells in serum-free medium with 0.1% BSA and vehicle (absolute ethanol, 0.47%, vol/vol) were used as control. (2) $CD4^+$ T lymphocytes (150 000 cells/well) were seeded in 96-well plates in their growth medium and stimulated for 1–6 days with P/I (1 μM/10 ng/ml), with or without the same drugs at the same concentration as for RPTEC. Cells in growth medium and vehicle (absolute ethanol, 0.47%, vol/vol) were used as control.

For both cell types, stimuli were added every other day. Cell viability was assessed each day by trypan blue (0.05% vol/vol solution in PBS) exclusion, as previously reported [21]. The number of viable cells was expressed as percentage of control RPTEC or P/I-activated $CD4^+$ T cells, respectively. Experiments were performed two to five times.

Statistical analysis

The statistical analyses were performed using SPSS 12.0 software package (SPSS for Windows 12.0; SPSS Inc,

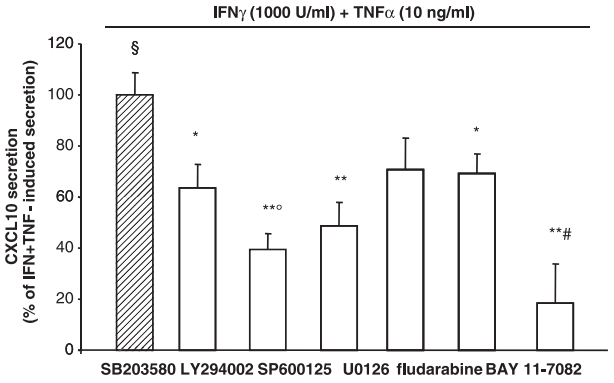

Figure 1 Effect of inhibition of different intracellular pathways on IFN-γ + TNF-α-induced CXCL10 secretion by RPTEC. CXCL10 protein secretion, virtually absent in control (not shown), significantly increased with IFN-γ + TNF-α (taken as 100%). NF-kB inhibitor BAY 11–7082 almost abrogated cytokine-induced CXCL10 secretion (81.69 ± 15.72% inhibition). CXCL10 secretion significantly decreased after blockade of p38 MAPK (SB203580, 36.36 ± 8.97% inhibition), PI3K (LY294002, 60.50 ± 6.26% inhibition), JNK (SP600125, 51.28 ± 9.46% inhibition) and Stat1 (fludarabine, 30.86 ± 7.71% inhibition). ERK blockade (U0126) showed no effect. §$P < 0.01$ vs. control; *$P < 0.05$, **$P < 0.01$ vs. IFN-γ + TNF-α-treatment; #$P < 0.05$ vs. SB203580-, U0126- or fludarabine-treatment; °$P < 0.05$ vs. fludarabine-treatment.

Chicago, IL, USA). The Kolmogorov–Smirnov test was used to test for normal distribution of the data. One-way analysis of variance (ANOVA) was applied and a *P*-value <0.05 was considered significant. The computer program ALLFIT (NIH, Bethesda, MD, USA) [31] was used for analysis of sigmoid dose-response curves to obtain estimates of IC_{50} of FK-506, MPA, elocalcitol and BXL-01-0029. Data were expressed as mean ± SE.

Results

The intracellular pathways involved in CXCL10 protein secretion have been investigated in RPTEC stimulated with IFN-γ + TNF-α in the presence of selective inhibitors of p38 MAPK (SB203580), PI3K (LY294002), JNK (SP600125), ERK (U0126), Stat1 (fludarabine) and NF-kB (BAY 11-7082).

The CXCL10 protein secretion, virtually absent in control (not shown), significantly increased with proinflammatory cytokines; almost all the inhibitors reduced IFN-γ + TNF-α-induced CXCL10 secretion (Fig. 1). In particular, NF-kB inhibition reduced the cytokine-induced CXCL10 secretion by over 80% ($P < 0.01$ vs. IFN-γ + TNF-α-treatment; $P < 0.05$ vs. SB203580-, U0126-, fludarabine-treatment); PI3K and JNK inhibition significantly reduced CXCL10 secretion as well (60.5 ± 6.26% and 51.28 ± 9.46%, respectively; $P < 0.01$ vs. IFN-γ + TNF-α-treatment; for LY294002 $P < 0.05$ vs. fludarabine-treatment) whereas p38 MAPK and Stat1 blockade inhibited CXCL10 secretion to a lesser extent (36.36 ± 8.97% and 30.86 ± 7.71%, respectively, $P < 0.05$ vs. IFN-γ + TNF-α-treatment); ERK inhibition exerted no significant effect.

The effect of the immunosuppressors on cytokine-induced CXCL10 secretion by RPTEC was assessed in cells incubated with IFN-γ + TNF-α, with or without a fixed dose of FK-506, MPA, elocalcitol, BXL-01-0029, Sir, H, CsA or MeP.

Tacrolimus (FK-506), MPA, elocalcitol and BXL-01-0029 significantly reduced cytokine-induced CXCL10 protein secretion (inhibition range 33–43%; $P < 0.05$, $P < 0.01$ vs. IFN-γ + TNF-α-treatment), while Sir, H, MeP and CsA exerted no effect (Fig. 2a). BXL-01-0029 exerted an inhibition significantly higher than FK-506 (42.58 ± 1.35% vs. 33.34 ± 2.77%, $P < 0.05$).

Real-time PCR mRNA analysis revealed that BXL-01-0029, similarly to MPA, significantly reduced cytokine-induced CXCL10 gene expression ($P < 0.01$ or $P < 0.05$ vs. IFN-γ + TNF-α-treatment, taken as 1, inset of Fig. 2 a), while FK-506 and elocalcitol did not.

The pharmacologic potency of the effective drugs was determined in RPTEC incubated with IFN-γ + TNF-α and increasing concentrations of FK-506, MPA, elocalcitol and BXL-01-0029.

Although the four drugs dose-dependently reduced the cytokine-induced CXCL10 secretion ($P < 0.05$, $P < 0.01$ vs. IFN-γ + TNF-α-treatment, Fig. 2b), VDR agonists were more potent than FK-506 and MPA. In particular, drug potency was as follows: MPA<FK-506 < elocalcitol<BXL-01-0029. BXL-01-0029 showed the greatest potency (BXL-01-0029: $-\log IC_{50} = 13.79 \pm 0.46$; elocalcitol: $-\log IC_{50} = 11.62 \pm 0.28$; FK-506: $-\log IC_{50} = 8.66 \pm 0.24$; MPA: $-\log IC_{50} = 6.01 \pm 0.29$; $P < 0.01$ each drug versus the other ones), as by ALLFIT program simultaneous fitting of the inhibitory curves [31]. We omitted the MPA-related highest dose (8.1×10^{-4} M) as it was cytotoxic and produced no feasible results.

For verifying a potential additive effect, different concentrations of BXL-01-0029 and FK-506 were simultaneously added to IFN-γ + TNF-α-stimulated RPTEC. BXL-01-0029 (10^{-8} M) significantly enhanced inhibitory effect of FK-506 (2.5×10^{-9}, 1.2×10^{-8}, 2.5×10^{-8} M) in relation to the induced CXCL10 protein secretion in RPTEC (Fig. 3, $P < 0.05$ vs. (2.5×10^{-8} M) FK-506-treatment); CXCL10 secretion was significantly reduced also after the combination of BXL-01-0029 at lower dosage (10^{-9} M) with FK-506 (2.5×10^{-8} M) [$P < 0.05$ vs. (2.5×10^{-8} M) FK-506-treatment].

Cell viability of RPTEC, treated from 1 to 6 days with a fixed dose of FK-506, MPA, elocalcitol and BXL-01-0029

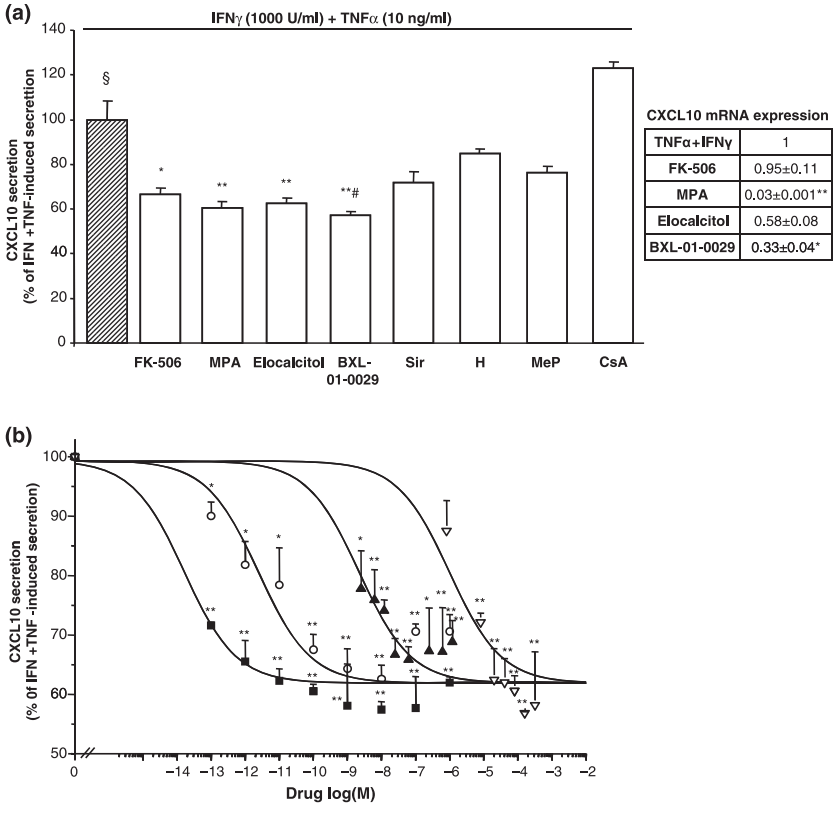

Figure 2 Response of cytokine-stimulated RPTEC to immunosuppressors. (a) FK-506 (2.5×10^{-8} M), MPA (8.1×10^{-5} M), elocalcitol (10^{-8} M) and BXL-01-0029 (10^{-8} M) significantly reduced IFN-γ + TNF-α-induced CXCL10 protein secretion (taken as 100%), while Sir (1.6×10^{-8} M), H (2.8×10^{-7} M), MeP (6.7×10^{-7} M) and CsA (2.1×10^{-7} M) were ineffective. *Inset*. MPA and BXL-01-0029 significantly decreased IFN-γ + TNF-α-induced CXCL10 gene expression (taken as 1). (b) BXL-01-0029 showed the greatest potency [BXL-01-0029 (closed squares): $-\log IC_{50} = 13.79 \pm 0.46$; elocalcitol (open circles): $-\log IC_{50} = 11.62 \pm 0.28$; FK-506 (closed triangles): $-\log IC_{50} = 8.66 \pm 0.24$; MPA (open triangles): $-\log IC_{50} = 6.01 \pm 0.29$; $P < 0.01$ each drug versus the other ones].§-$P < 0.01$ vs. control; $**P < 0.01$, $*P < 0.05$ vs. IFN-γ + TNF-α-treatment; $\#P < 0.05$ vs. FK-506-treatment.

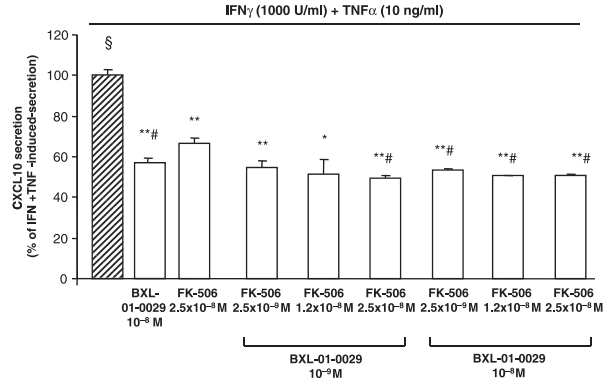

Figure 3 Effect of BXL-01-0029 and FK-506 combination on IFN-γ + TNF-α-induced CXCL10 secretion by RPTEC. BXL-01-0029 combined with FK-506 showed additive inhibitory effects onto CXCL10 protein secretion by RPTEC. The addition of BXL-01-0029 (10^{-8} M) significantly enhanced FK-506 (2.5×10^{-8} M)-induced inhibition and allowed to lower FK-506 doses (2.5×10^{-9}, 1.2×10^{-8} M) to reach the same inhibitory effect onto IFN-γ + TNF-α-induced CXCL10 secretion (taken as 100%). The same result was obtained with the addition of BXL-01-0029 at lower dose (10^{-9} M) to FK-506 (2.5×10^{-8} M). §$P < 0.01$ vs. control; $**P < 0.01$, $*P < 0.05$ vs. IFN-γ + TNF-α-treatment; $\#P < 0.05$ vs. FK-506 (2.5×10^{-8} M)-treatment.

was assessed. FK-506, elocalcitol and BXL-01-0029 did not affect viable cell number versus control (taken as 100%, not shown). In contrast, MPA significantly reduced viable cells starting from the third day of treatment ($P < 0.05$, $P < 0.01$ vs. control or other drug treatment; Fig. 4a).

Drug toxicity was also evaluated on $CD4^+$ T cells, activated with P/I (1 μM/10 ng/ml) and treated as in the case of RPTEC. Neither BXL-01-0029, as already stated in [21], nor elocalcitol exerted any significant effect on cell viability of P/I-activated $CD4^+$ T cells (taken as 100%, not shown, Fig. 4b). FK-506 and MPA reduced viable $CD4^+$ T cells 4 and 3 days after treatment, respectively ($P < 0.05$, $P < 0.01$ vs. P/I-activated cells). Although MPA effect on $CD4^+$ T-cell viability has been previously demonstrated [21], in this study the statistical significance was reached at 3 days rather than 4 days after treatment, resulting from the increased number of experimental observations.

Discussion

Both elocalcitol and BXL-01-0029 significantly reduced IFN-γ + TNF-α-induced CXCL10 protein secretion in RPTEC; the conventional immunosuppressors FK-506 and MPA also targeted CXCL10 secretion, while Sir, H, MeP and CsA were found to be ineffective. BXL-01-0029 and MPA significantly reduced also the CXCL10 gene expression. BXL-01-0029 reduced CXCL10 secretion in RPTEC with the greatest potency. The simultaneous

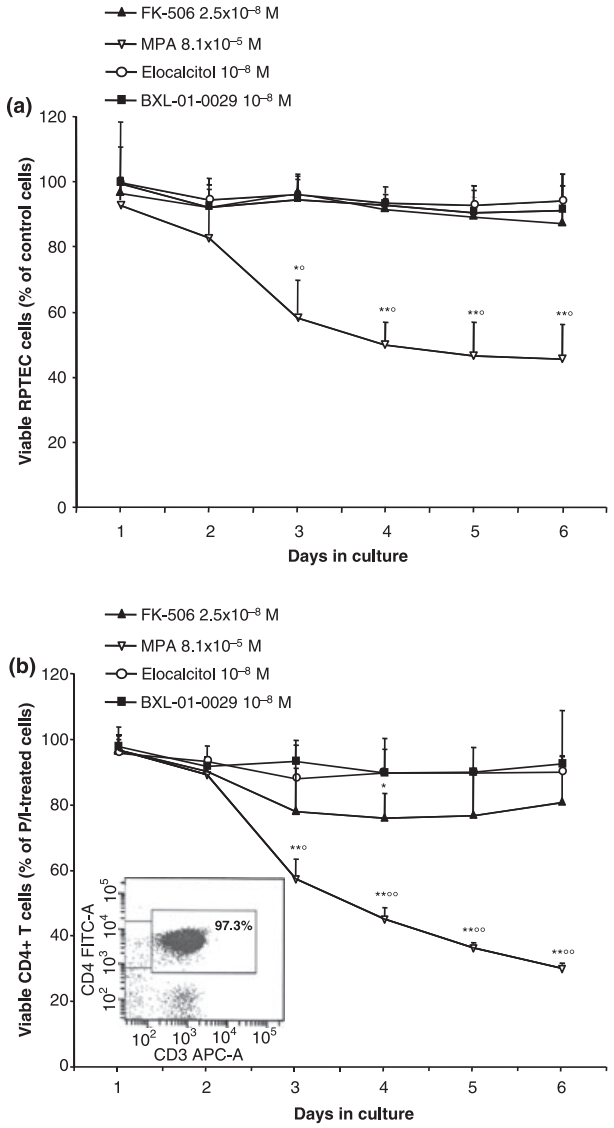

Figure 4 Effect of FK-506, MPA, elocalcitol and BXL-01-0029 on RPTEC and $CD4^+$ T-cell viability.(a) FK-506 (2.5×10^{-8} M, closed triangles), elocalcitol (10^{-8} M, open circles) and BXL-01-0029 (10^{-8} M, closed squares) did not significantly affect RPTEC viability versus control, taken as 100%, at variance with MPA (8.1×10^{-5} M). (b) Cell viability of P/I-activated $CD4^+$ T cells, taken as 100%, was unaffected by BXL-01-0029 (closed squares) and elocalcitol (open circles); FK-506 (closed triangles) and MPA (open triangles) significantly reduced viable cells. Drug concentrations were the same as used in RPTEC. *Inset*. $CD4^+$ T-cell purity was 97.3%. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control cells or vs. P/I-treated cells; $^{\circ\circ}P < 0.01$, $^{\circ}P < 0.05$ vs. FK-506-, elocalcitol- and BXL-01-0029-treatment.

presence of BXL-01-0029 and FK-506 showed additive effect onto cytokine-induced CXCL10 protein secretion in RPTEC. Neither BXL-01-0029 nor elocalcitol affected RPTEC viability, just as in the case of FK-506, whereas MPA reduced viable cell number.

During allograft dysfunction following kidney transplant, the decline of renal function correlates with the progressive tubulointerstitial injury and renal fibrosis [1,32–35].

Tubular damage, mostly driven by interstitial Th1-type leukocyte accumulation, is perpetuated by high chemokine production at the site of inflammation. A chemokine gradient between the area of inflammation and neighboring vascular endothelium is responsible for further interstitial leukocyte deposit, thus establishing a self-inflammatory loop. In particular, CXCL10–CXCR3 interactions appear to play an important role in the pathogenesis of graft failure caused by rejection in multi-organ models [5,36–38]. Notably, CXCL10 intragraft production has been shown to initiate alloresponse in AR in the context of heart transplantation [39]. Furthermore, the association between elevated pretransplant serum levels of CXCL10 and the onset of AR in kidney as well as heart transplantations, chronic allograft nephropathy and kidney graft failure has been documented [7,40,41].

Thus, interfering with CXCL10–CXCR3 axis might be essential in the resolution or progression of renal disease.

Accordingly, the observation that elocalcitol and BXL-01-0029 – the latter with the highest potency – reduced CXCL10 protein secretion induced by proinflammatory cytokines in RPTEC appears interesting.

Vitamin D receptor agonists have been shown to inhibit renal inflammation and interstitial fibrosis in experimental models [18,42–45] and to reduce proteinuria and all-cause mortality [12–17] in chronic renal disease patients. Beyond the inhibition of renin–angiotensin system [18,45–49], the beneficial effect of VDR activators seems attributable to their intrinsic anti-inflammatory properties [50,51]. VDR ligands, in fact, inhibit Th1 and Th17 cells and their related cytokines [52] and also chemokine production by target tissue cells [53–56], particularly via inhibition of NF-kB activation [20,21,23,55,57]. Notably, NF-kB inhibition could be an important strategy to prevent the progression of renal fibrosis [58,59]. As previously shown, elocalcitol and BXL-01-0029 reduced CXCL10 protein secretion in target tissue cells and in $CD4^+$ T cells [20,21]. Herein, we documented that both VDR agonists can impair CXCL10 protein secretion by RPTEC as well. Although we have previously shown the capability of elocalcitol and BXL-01-0029 to interfere with TNF-α and IFN-γ pathways in human cardiomyocytes and thyrocytes, further investigations need to be conducted to clarify their mechanism of action onto RPTEC. Consistent with our experiments with selective inhibitors, NF-kB, JNK and PI3K seem the pathways most involved in proinflammatory cytokine-induced CXCL10 protein secretion by RPTEC, while p38 MAPK and Stat-1 appear to be engaged to a lesser extent.

Based on the data of our previous results in different cell types, elocalcitol and BXL-01-0029 exert their effect impairing NF-kB, Stat-1 and PI3K cellular pathways [20,21,60]. Therefore, we could speculate that blockade of such intracellular pathways could explain, in part, the mechanism of action exerted by VDR agonists in RPTEC. Furthermore, the finding that BXL-01-0029 is the most potent drug and is able to significantly raise FK-506 inhibitory effect onto CXCL10 protein secretion in RPTEC seems intriguing. Notably, drug combination allows lowering of the dosage of both drugs to reach the same inhibition extent. The anti-inflammatory potential of FK-506 has been recently explained in association with NF-kB suppression in renal tubular epithelial cells [61]. BXL-01-0029, as previously mentioned, blunted NF-kB translocation in cardiomyocytes while impairing CXCL10 secretion [21], in consonance with the observation that its active agent, BXL-219, is a NF-kB inhibitor through NF-kBp65 nuclear translocation arrest and IkB-α transcription up-regulation [55]. Therefore, FK-506 and BXL-01-0029 synergic effect might be partially attributable to the simultaneous NF-kB translocation blockade by both drugs, in addition to CXCL10 gene expression inhibition by BXL-01-0029. Previous data showed that VDR activators, by lowering CsA therapeutic doses, reduced the risk of AR [10] or chronic nephrotoxicity [62]. More recently, protective efficacy of paracalcitol, a VDR activator, combined with trandolapril, an angiotensin-converting enzyme inhibitor, has been reported in an obstructive nephropathy mouse model [19]. Those observations could be relevant, as conventional immunosuppressors exert their therapeutic effect at doses close to the toxic range, whereas elocalcitol and BXL-01-0029 impaired Th1-mediated inflammatory response by targeting CXCL10, affecting viability neither in RPTEC, as shown herein, nor in lymphocytes, as reported here and elsewhere [21]. Similarly, FK-506 did not affect cell viability, according to previous data showing no cytotoxicity at low concentrations in tubular renal cells [63]. MPA, previously reported to abrogate tubular renal cell proliferation [64], in our study was found to reduce RPTEC viability, in line with a recent report [65].

The variety of current immunosuppressive regimens confirms the absence of dominant therapeutic protocol(s), although calcineurin inhibitors, despite their nephrotoxicity, remain the mainstay of therapy in kidney transplantation [66]. The second challenge in post-transplant therapy is to avoid chronic nephrotoxicity [67]. FK-506 appears to be less nephrotoxic than CsA, which may explain why many centers have adopted FK-506-based immunosuppressive regimens [68].

Future *in vivo* investigations are necessary to verify whether VDR analogs could prevent AR of transplanted kidney. Previous results are encouraging, as in humans vitamin D supplementation favored immune tolerance towards liver allograft [69]. Furthermore, elocalcitol inhibited in animals chronic aortic graft rejection and BXL-219 impaired Th1-mediated inflammation [11,55]. Interestingly, elocalcitol showed excellent safety and tolerability in benign prostate hyperplasia patients [70].

In conclusion, our data suggest that the use of VDR agonists as therapeutic agents might contribute to management of kidney transplant rejection. Further attention need to be dedicated to BXL-01-0029 as a novel potential dose-reducing drug for conventional immunosuppressors and future studies should focus on its effect onto CXCL9 secretion as well, as this chemokine has emerged as a useful parameter to predict AR episodes in transplanted kidneys and graft outcome [71]. Multitarget therapy – a combination of different drug classes intended to decrease therapeutic doses and minimize side-effects – with the ability to impair chemokine(s) trafficking in different cell types might indeed help to improve kidney transplant recipient management.

Authorship

CS and MS: performed research, analyzed data. BM and EB: collected data. LA: contributed important reagents. MS and PR: designed research and contributed important reagents. CC: designed research and wrote the paper.

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