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Dual blockade of the pro-inflammatory chemokine CCL2 and the homeostatic chemokine CXCL12 is as effective as high dose cyclophosphamide in murine proliferative lupus nephritis

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ABSTRACT

Induction therapy of proliferative lupus nephritis still requires the use of unselective immunosuppressive drugs with significant toxicities. In search of more specific drugs with equal efficacy but fewer side effects we considered blocking pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) and homeostatic chemokine stromal cell-derived factor-1 (SDF-1/CXCL12), which both contribute to the onset and progression of proliferative lupus nephritis yet through different mechanisms. We hypothesized that dual antagonism could be as potent on lupus nephritis as the unselective immunosuppressant cyclophosphamide (CYC). We estimated serum levels of CCL2 and CXCL12 in patients with SLE (n = 99) and compared the results with healthy individuals (n = 21). In order to prove our hypothesis we used L-enantiomeric RNA Spiegelmer® chemokine antagonists, i.e. the CCL2-specific mNOX-E36 and the CXCL12-specific NOX-A12 to treat female MRL/lpr mice from week 12 to 20 of age with either anti-CXCL12 or anti-CCL2 alone or both. SLE patients showed elevated serum levels of CCL2 but not of CXCL12. Female MRL/lpr mice treated with dual blockade showed significantly more effective than either monotherapy in preventing proteinuria, immune complex glomerulonephritis, and renal excretory failure and the results are at par with CYC treatment. Dual blockade reduced leukocyte counts and renal IL-6, IL-12p40, CCL-5, CCL-2 and CCR-2 mRNA expression. Dual blockade of CCL2 and CXCL12 can be as potent as CYC to suppress the progression of proliferative lupus nephritis probably because the respective chemokine targets mediate different disease pathomechanisms, i.e. systemic autoimmunity and peripheral tissue inflammation.

1. Introduction

Control of human proliferative lupus nephritis (LN) requires potent immunosuppression with a combination of steroids and either cyclophosphamide (CYC) or mycophenolate mofetil (MMF) [1,2]. Each of these combinations is associated with significant morbidity and mortality. For example, in the Aspreva Lupus Management Study trial MMF caused serious adverse effects in 27.7% and treatment-related death in 4.9% and CYC in 22.8% and 2.8% of treated patients, respectively [3]. The most serious adverse effects and deaths were related to infections due to the unspecific immunosuppressive effects of CYC and MMF [3]. Novel more specific drugs may help to reduce the toxicity of current treatment protocols [4].

Cytokine antagonism is a powerful strategy to prevent tissue damage in chronic inflammation [5,6]. Beyond cytokines and interleukins, CC-chemokines represent potential targets for specific antagonism because CC-chemokines mediate immune cell activation and their recruitment to sites of inflammation [7–10]. CCL2, also known as monocyte-chemoattractant protein-1 and its chemokine receptor CCR2 promote LN by driving intrarenal inflammation via the recruitment and activation of pro-inflammatory leukocyte subsets [11,12]. CCL2- or CCR2-deficient MRL/lpr mice are protected from proliferative LN [13,14], hence, several groups tried to block CCL2 or CCR2 with suitable antagonists in mice with experimental SLE [15,16]. The results validated the CCL/CCR2 axis as a target in general, but the chosen approaches-gene transfer causing irrepressible antagonist production and injection of

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antagonist-producing transfected calls do not represent applicable therapeutic strategies in humans.

To overcome these limitations we have recently proposed an alternative strategy to block CCL2 with a CCL2-binding mirror-image RNA oligonucleotide, a so-called Spiegelmer® [17]. Spiegelmers are nuclease-resistant RNA aptamers that can bind and inhibit target molecules conceptually similar to monoclonal antibodies [18]. Anti-CCL2 Spiegelmer mNOX-E36 treatment after onset of proliferative LN significantly suppressed disease progression in female MRL/lpr mice as compared to treatment with a non-active control Spiegelmer [19]. In a subsequent study we demonstrated that using mNOX-E36 in combination of low-dose CYC was as effective as high dose CYC albeit avoiding systemic T cell ablation and myelosuppression as markers of severe systemic immunosuppression [20]. In the present study we questioned if another more specific agent could even replace low-dose CYC in such a combination drug regime. Ideally, such a drug should have a different mechanism-of-action than mNOX-E36.

The homeostatic chemokine stromal cell-derived factor-1 (SDF-1), also named CXCL12, is constitutively expressed in many tissues where it regulates neutrophil trafficking as well as the homing and function of stem or progenitor cells [21]. Inside the kidney, CXCL12 is constitutively expressed by glomerular podocytes and CXCL12 blockade was shown to prevent podocyte loss and scarring in diabetic kidney disease [22]. In addition, we have previously shown that combined blockade of the targets CCL2 and CXCL12 had additive protective effects on diabetic kidney injury; these results are based on the target's different modes-of-action in intrarenal inflammation, glomerular injury, and repair [23]. CXCL12 blockade also controls LN in NZBW mice [24]. Therefore, we speculated that dual blockade of CCL2 and CXCL12 may also be a potent treatment of LN, potentially as potent as high dose CYC.

2. Material and methods

2.1. CCL2 and CXCL12 antagonistic Spiegelmers

The sequences of the CCL2 antagonistic Spiegelmer mNOX-E36 (5′-GGCGACA UUGGUUGGGCAUGAGGCGAGGCCCUUUGAUGAAUCCGCGG CCA-3′) and the CXCL12 antagonistic Spiegelmer NOX-A12 (5′-GCGUGGUGUGAUCUAGAUGUAUUGG CUGAUCCUAGUCAGGUACGC-3′) were obtained from in vitro selection experiments that were essentially carried out as described [17,34]. Both mNOX-E36 and NOX-A12 bind to their targets with subnanomolar affinities. Chemokine inhibition was determined for each Spiegelmer using leukocyte chemotaxis assays as described [17,19]. All Spiegelmers have been tested already as single agents by our group as described [23,35].

2.2. Animal studies

Eight-week-old female MRL/lpr mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept under pathogen-free and normal housing conditions in a 12-h light and dark cycle. At the age of 12 weeks, proteinuria was assessed and only proteinuric mice (A/C ratio > 2) were randomized and divided into five groups (n = 10–12). Each of these groups received either vehicle (5% glucose) or subcutaneous injections of 13.4 mg/kg NOX-A12, 14.4 mg/kg mNOX-E36, combination of both or in 5% glucose every other day and cyclophosphamide injected 30 mg/kg i.p. once in a week. The treatment dose and injection intervals were comparable to our mouse studies with similar formulations of Spiegelmers targeting these chemokines [19,20,22,23,35]. Treatment was continued for 8 weeks. Tissues were harvested for histopathological evaluation at the end of the treatment period. Blood and urine samples were obtained at monthly intervals for the estimations of urinary albumin (ELISA: Bethyl Labs, Montgomery, TX, USA) as well as serum and urinary creatinine (Jaffé reaction: DiaSys Diagnostic Systems, Holzheim, Germany). All experiments were performed according to German animal protection laws and had been approved by the local government authorities.

2.3. Histopathology

Kidneys were harvested from each mouse and were fixed in 10% formalin in PBS and embedded in paraffin. Sections of 2 μm were stained with periodic acid-Schiff reagent. The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis [25,36]. Immunostaining was performed as described [37] using the following primary antibodies: IgG (Invitrogen, Carlsbad, CA) and Mac-2 (Cedarlane, Burlington, NC, USA). Stains were assessed by light microscopy in 15 glomeruli per section by a blinded observer.

2.4. RNA preparation and real-time quantitative (TaqMan) RT-PCR

Total RNA was isolated from kidneys using Qiagen RNA extraction kit (Hilden, Germany) following the manufacturer's instructions. After quantification RNA quality was assessed using agarose gels. From isolated RNA, cDNA was prepared using reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA). Real time-PCR was performed using SYBR Green PCR master mix and was analyzed with a Light Cycler 480 (Roche) as described [38] using the genes listed in Table 2. All gene expression values were normalized using 18S RNA as a house keeping gene. All primers used for amplification were from Metabion (Martinsried, Germany).

2.5. Flow cytometry

Flow cytometry of the kidney cell suspensions was performed as described [39] using the following conjugated antibodies: PE anti-CD45,

Table 1
Clinical and demographic features of SLE patients and healthy control subjects.

	Control n = 21	SLE n = 99
Female, n (%)	18 (85)	82 (82)
Male, n (%)	3 (14)	17 (17)
Age (years)	48.4 ± 14.1	46.4 ± 17.2
SLEDAI score > 4, n (%)	–	79 (79)
SLICC/ACR damage index >1, n (%)	–	79 (79)
Leukopenia, n (%)	–	23 (23)
Thrombocytopenia, n (%)	–	20 (20)
Fatigue, n (%)	–	66 (66)
Arthralgia, n (%)	–	79 (79)
Jaccoud's arthropathy, n (%)	–	34 (34)
Malar rash, n (%)	–	31 (31)
Pericarditis, n (%)	–	20 (20)
Pleuritis, n (%)	–	21 (21)
Venous thrombosis, n (%)	–	13 (13)
Lupus nephritis, n (%)	–	43 (43)
ISN/RPS class I, n (% of all LN)	–	1 (1.2)
ISN/RPS class II, n (% of all LN)	–	9 (9.6)
ISN/RPS class III, n (% of all LN)	–	11 (11.9)
ISN/RPS class IV, n (% of all LN)	–	21 (21.5)
ISN/RPS class V, n (% of all LN)		4 (4.7)
ISN/RPS class VI, n (% of all LN)		1 (1.2)
CKD, n (%)	–	30 (30)
CKD 1, n (% of all CKD)	–	15 (15)
CKD 2, n (% of all CKD)	–	1 (1)
CKD 3, n (% of all CKD)	–	8 (8)
CKD 4, n (% of all CKD)	–	4 (4)
CKD 5, n (% of all CKD)	–	3 (3)
Hemolytic anemia, n (%)	–	18 (19)
Antinuclear antibodies, n (%)	–	88 (88)
Anti-dsDNA IgG, n (%)	–	46 (56)

LN = lupus nephritis, CKD = chronic kidney disease, ISN = International Society of Nephrology, RPS = Renal Pathology Society, SLEDAI = Systemic Lupus Erythematosus activity index, ACR = American College of Rheumatology, SLICC = Systemic Lupus International Collaborating Clinic.