Mini Review

The role of a chemokine receptor, CCR2, in suppressing the development of arthritis in IL-1 receptor antagonist-deficient mice

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Rheumatoid arthritis (RA) is a chronic inflammatory disease and is characterized by infiltration of macrophages and neutrophils into the joint space. A chemokine receptor, CC chemokine receptor (CCR) 2, is expressed on macrophages infiltrating into the synovium of RA patients, but the roles of CCR2-mediated signals in RA still remain controversial. We demonstrated that ablation of *Ccr2* gene aggravated the polyarthritis, which develops in interleukin-1 receptor antagonist (IL-1ra)-deficient mice. This is associated with augmented neutrophil infiltration and osteoclastogenesis. Due to the important role of the CCR2-mediated signals in the egress of monocytes from bone marrow, *Ccr2* gene ablation resulted in increased number of monocytes, a precursor of osteoclasts, in bone marrow of IL-1ra-deficient mice. Intraarticular neutrophils expressed the receptor of activator of NF-κB ligand (RANKL) and a disintegrin and metalloproteinase (ADAM) 8, the factors which are crucially involved in osteoclast formation. Additional blockade of neutrophil infiltration decreased the numbers of osteoclasts and attenuated arthritis in IL-1ra-CCR2-double deficient mice. These observations revealed that CCR2-mediated signals can modulate arthritis development and progression by suppressing the egress of monocytes, a precursor of osteoclasts and promoting the infiltration of neutrophils, a rich source of osteoclastogenic factors.

Rec.12/2/2011, Acc.2/27/2012, pp124-131

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Key words cytokines, chemokines, arthritis, IL-1ra, osteoimmunology

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder of synovium, cartilage, and bone, of unknown etiology, and is characterized by infiltration of various inflammatory cells, such as monocytes/macrophages, neutrophils, and lymphocytes into the synovium and periarticular space[1]. The important pathogenic roles of tumor necrosis factor (TNF)-α has been proven by the observation that TNF antagonists induce a remarkable response rate in RA compared with conventional therapy[2]. Moreover, monocytes/macrophages are a rich source of TNF-α and their numbers in the synovium correlate well with clinical symptoms and joint damage in RA[3]. Thus, inhibition of synovial macrophage accumulation may be able to prevent disease progression in RA.

Chemokines are a large superfamily of small proteins that can regulate the trafficking, accumulation and activation of various types of cells, particularly leukocytes[4]. Chemokine receptors are G protein-coupled receptors and each chemokine receptor is expressed by distinct cell types. Thus, the identity of expressed chemokine receptors can determine the responsiveness of leukocytes to particular chemokines[5]. Monocytes/macrophages abundantly express several chemokine receptors including CCR2, CCR5, and CX3CR1. Moreover, inflammatory monocytes/macrophages, which infiltrate into inflammatory sites, express CCR2[4]. These observations prompted us to investigate the roles of the CCR2-mediated signals in arthritic joint lesions. We did this using IL-1 receptor antagonist (IL-1ra)-deficient mice, which spontaneously develop polyarthritis resembling human RA. Here, we will discuss the potential roles of the CCR2-mediated signals in spontaneously developing arthritis in IL-1ra-deficient mice, after briefly summarizing the roles of IL-1 and IL-1ra in RA.

Potential involvement of IL-1α, IL-1β and IL-1ra in RA

The IL-1 family includes two agonists, IL-1α and IL-1β, and one antagonist, IL-1ra. The genes encoding three proteins reside in adjacent position in 2q13 in humans[6]. IL-1α and IL-1β are first synthesized as precursors without an apparent signal peptide by various types of cells including neutrophils, monocytes/macrophages and synovial lining cells. These precursors of IL-1α and IL-1β are cleaved to generate mature forms with full biological activities, mainly, but not exclusively, by calpain and caspase-1, respectively[6]. In contrast, IL-1ra protein is transcribed as a protein with a signal peptide[7].

Although IL-1α, IL-1β, and IL-1ra show at most 30 % sequence homology with each other, they exhibit a similar pleated sheet structure and bind to IL-1 receptor at the same site with a similar affinity[7]. Binding of either IL-1α or IL-1β induces the association of IL-1 receptor with IL-1 receptor accessory protein, the molecule which is indispensable for intracellular signal transduction[7]. Eventually, IL-1α and IL-1β induce monocytes/macrophages, lymphocytes, synovial cells, and endothelial cells to produce various cytokines, chemokines, and inflammatory mediators including IL-1 itself, TNF-α, IL-17, IL-8, matrix metalloproteinases (MMPs), and receptor activator of nuclear factor-κB ligand (RANKL)[8]. In contrast, the binding of IL-1ra cannot induce the association between IL-1 receptor and IL-1 receptor accessory protein[7]. As a consequence, IL-1ra cannot generate intracellular signals, thereby acting as a natural competitive antagonist of IL-1α and IL-1β. Thus, the balance between IL-1ra and IL-1 levels in local tissues determines the relative physiologic or pathological effects of IL-1.

Evidence has clearly shown the presence of IL-1 in the synovial fluid of RA and the localization of IL-1 in the lining layer of rheumatoid synovium, particularly macrophages at the pannus-cartilage[9]. IL-1 can induce proteoglycan degradation and inhibit proteoglycan synthesis by chondrocytes, and enhance collagen degradation in cartilage[6]. IL-1α-transgenic mice consistently exhibited an inflammatory arthritis similar to RA, while injection of neutralizing anti-IL-1 antibodies reversed abnormalities present in collagen-induced arthritis (CIA)[10,11]. These observations encouraged clinical trials using IL-1ra, as a therapeutic drug for RA. Combination therapy consisting of methotrexate and human IL-1ra has been shown to provide greater clinical benefits in RA than methotrexate alone[12], but most studies failed to provide evidence to indicate the apparent clinical benefits of IL-1ra[13]. This may be due to the short half-life of exogenously administered IL-1ra. Alternatively, high concentrations to achieve more than 100-fold or greater levels of IL-1ra are necessary to inhibit the biological effects of IL-1 on target cells.

IL-1ra is localized in rheumatoid synovium, particularly in the cells lining the joint and in the sublining area in a perivascular distribution in macrophages, but the levels of IL-1ra are not sufficient to inhibit the injurious effects of IL-1[7].

However, continuous IL-1ra infusion to achieve sustained blood levels of IL-1ra did alleviate arthritis symptoms in CIA and antigen-induced arthritis[7]. IL-1ra-transgenic over-expressing mice exhibited less severe CIA, whereas IL-1ra-deficient mice developed more aggressive CIA[14,15]. Moreover, analysis of human IL-ra (gene name, *Il1rn*) gene polymorphisms revealed that homozygocity of *Il1rn*2* was associated with lower plasma IL-1ra levels and with an increased number of affected articular areas in RA patients[16,17]. These observations implicate IL-1ra as a protective factor for RA.

IL-1ra-deficient mice on a BALB/c background spontaneously develop polyarthritis[18]. Marked synovial and periarticular inflammation are observed in the affected joints, together with articular erosion caused by invasion of granulation tissues. Moreover, there is marked proliferation of synovial lining cells and invading inflammatory cells engage in forming a pannus. There is considerable bone erosion and this is associated with replacement of the bone matrix with fibroblastic cells, together with marked activation of osteoclasts[18]. These histological features resemble those observed in RA patients. Thus, IL-1ra-deficient mice can be a good animal model for the elucidation of molecular and cellular mechanisms underlying polyarthritis in RA patients.

Genetic studies revealed that the spontaneous arthritis of IL-1ra-deficient mice is highly dependent on non-major histocompatibility complex genes[19]. Moreover, autoimmunity to type II collagen is not the major disease-inducing event in IL-1ra-deficient mice[19]. In contrast, the pathogenic roles of TNF-α and IL-17 have been proven by the observations that the ablation of either of these genes reduces the evidence of arthritis in IL-1ra-deficient mice[20, 21]. Moreover, gene expression profile studies revealed that the mRNA expression of various chemokines including monocyte chemoattractant protein (MCP)-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7, is enhanced in the joints of both IL-1ra-deficient mice[22]. Furthermore, the joints of these mice exhibit enhanced mRNA expression of CCR2, a receptor for CCL2, CCL7, and CCL8[22]. These observations suggest the potential involvement of CCR2-mediated signals in polyarthritis in these mouse models.

The CCL2-CCR2 axis in polyarthritis of IL-1ra-deficient mice

Monocytes/macrophages abundantly express several chemokine receptors, particularly CCR2. Synovial levels of CCL2, a ligand for CCR2, is markedly increased in RA joints[23], and genetic and pharmacological interference with the receptor has been shown to reduce disease activity in various animal models of RA[24-31]. However, Brühl et al. demonstrated that the CCR2 blockade had either beneficial or detrimental effects on CIA, depending on the phase of the disease[32]. Moreover, in some models, blockade of the CCL2-CCR2 axis had either no effect on disease[33] or even aggravated the disease under some conditions (Table 1)[32, 34-37]. Furthermore, several clinical studies demonstrated that either anti-CCL2 or anti-CCR2 antibody failed to improve the symptoms when given to RA patients[38, 39]. The failure in improvement may arise from the incapability of these antibodies to inhibit the infiltration of $CD3^+$ lymphocytes, $CD22^+$ lymphocytes and $CD68^+$ macrophages into synovial tissue[38, 39]. Nevertheless, the roles of the CCL2-CCR2 axis in RA remain an open question.

In addition to CCL2, several additional CC chemokines can bind CCR2. Moreover, potent neutralizing anti-mouse CCR2 antibody was not available. These circumstances prompted us to generate mice lacking both *IL-1ra* and

Table 1 Summary of animal studies examining effects of CCL2/CCR2 axis blockade on arthritis models

Species	Model	Intervention	Clinical outcome	Ref.
mouse	MRL/lpr arthritis	MCP-1(9-76)	improved	24
rat	CIA	anti-MCP-1 Ab	improved	25
rat	SCWA	anti-MCP-1 Ab	improved	26
rabbit	LPSA or MSUA	anti-MCP-1 Ab	improved	27
mouse	Lyme arthritis	CCR2 KO	slightly aggravated	34
mouse	CIA	anti-CCR2 Ab	improved (*)	32
			aggravated (**)	
mouse	CIA	CCR2 KO	aggravated	35
rat	AIA	CCR2 antagonist	improved	28
mouse	CIA	anti-CCR2 Ab (low dose)	improved	36
		anti-CCR2 Ab (high dose)	aggravated	
rat	AIA	P8A-MCP-1	improved	29
mouse	CIA	MCP-1(6-76, GMME1)	improved	30
mouse	ABIA	CCR2 antagonist	no effect	33
mouse	IL-1ra KO	CCR2 KO	aggravated	37
rat	CIA and AIA	MCP-1 antagonist	improved	31

Abbreviations used in the table; CIA, collagen-induced arthritis; SCWA, streptococcal cell wall-induced arthritis; LPSA, lipopolysaccharide-induced arthritis; MSUA, monosodium urate sodium-induced arthritis; ABIA, anti-collagen antibody-induced arthritis; AIA, adjuvant-induced arthritis. *Ab was administrated in initiation phase, **Ab was administrated in progression phase.

CCR2 genes (designated as IL-1ra-CCR2-doubly-deficient mice) by mating IL-1ra-deficient mice and CCR2-deficient mice on a BALB/c background[37], in order to address the roles of the CCL2-CCR2 axis, particularly CCR2-expressing cells in polyarthritis of IL-1ra-deficient mice. The incidence and the time of the onset of the disease were comparable between IL-1ra-deficient and IL-1ra-CCR2-doubly-deficient mice, but after 16 weeks of age, IL-1ra-CCR2-doubly-deficient mice had higher arthritis scores, with extensive swelling and severe ankylosis in multiple joints, compared with IL-1ra-deficient mice[37]. Moreover, IL-1ra-CCR2-doubly-deficient mice exhibited exaggerated intraarticular infiltration of neutrophils, but not macrophages, compared with IL-1ra-deficient mice. Furthermore, signs of joint inflammation, such as cartilage damage, bone erosion, and cell infiltration were augmented in IL-1ra-CCR2-doubly-deficient mice at ages of 16 weeks and older compared with IL-1ra-deficient mice. In contrast, CCR2-deficient mice on a BALB/c background did not develop any signs of arthritis until 1 year of age[37]. Thus, CCR2-mediated signals have a protective role in the spontaneous polyarthritis of IL-1ra-deficient mice.

Monocytes/macrophages, particularly inflammatory infiltrating monocytes/macrophages, express mainly CCR2[4]. Thus, it is tempting to assume that CCR2-mediated signals can promote intraarticular inflammatory macrophage infiltration. Supporting this notion, a low molecule weight CCR2 antagonist decreased macrophage infiltration into joints and improved arthritis in rat adjuvant arthritis and mouse CIA (Table 2)[29, 30]. In contrast, IL-1ra-CCR2-doubly-deficient mice exhibited similar levels of intraarticular monocyte/macrophage infiltration as IL-1ra-deficient mice[37]. Similar observations were obtained in CCR2-deficient mice, which were induced to develop CIA[35]. Expression of several macrophage-tropic chemokines, macrophage inflammatory protein (MIP)-1α/CCL3 and regulated upon activated T cells (RANTES)/CCL5 were enhanced in CIA of CCR2-deficient mice[35] (Table 2). Likewise, monocyte chemotaxis induced by RA synovial fluid was consistently inhibited by the antibody against CCR1, a receptor for CCL3 and CCL5, but not by antibody against CCR2[40].

Intraarticular neutrophil infiltration and intraarticular CXCL1 and CXCL2 levels were increased to a greater extent in IL-1ra-CCR2-doubly-deficient mice, than IL-1ra-deficient mice[37]. Because the IL-1 axis is a potent inducer of CXCL1 and CXCL2, the lack of IL-1ra may account for enhanced CXCL1 and CXCL2 expression in IL-1ra-CCR2-doubly-deficient mice. Moreover, neutrophils were a major source of CXCL1 and CXCL2 in the joints of IL-1ra-CCR2-doubly-deficient mice, suggesting the existence of a positive feedback mechanism between neutrophil infiltration and these chemokines. Supporting this notion, an antibody against CXCR2, a specific receptor for CXCL1 and CXCL2, markedly reduced intraarticular neutrophil infiltration and eventually decreased the arthritis scores in IL-1ra-CCR2-doubly-deficient mice[37]. However, it remains unknown whether either CCL2- or CCR2-antagonist can have similar effects on intraarticular neutrophil infiltration and bone marrow composition in RA patients because hitherto conducted clinical trials did not examine the effects of these inhibitors on these aspects[38,39].

The blockade of the CCL2-CCR2 axis also resulted in enhanced neutrophil migration and overexpression of neutrophil-tropic chemokines, CXCL1 and CXCL2, in CIA model[35]. Likewise, tumor-bearing CCR2-deficient mice exhibited enhanced CXCL1 and CXCL2 expression, com-

Table 2 Effect of the CCL2/CCR2 axis blockade on various aspects of arthritis models

Species	rat	mouse	mouse	mouse	mouse
Model	AIA	CIA	ABIA	CIA	IL-1ra KO
Intervention	P8A-MCP-1	MCP-1 (6-76, GMME1)	CCR2 antagonist	CCR2 KO	CCR2 KO
Clinical outcome	improved	improved	no effect	aggravated	aggravated
Cellular infiltrate in joints					
T cells	?	↓	?	↑	→
B cells	?	?	?	↑	→
Macrophages	↓	↓	↓	↑	→
Neutrophils	?	↓	→	↑	↑
Cytokine profile					
IL-1	↓	?	?	?	↑
IL-6	?	↓	?	↑	→
IL-17	?	↓	?	?	→
TNF-α	↓	↓	?	→	→
Chemokine profile					
CXCL1	?	?	→	?	↑
CXCL2	?	?	?	↑	↑
CCL2/MCP-1	?	↑	↑	↑	→
CCL3/MIP-1α	?	?	?	↑	→
CCL4/MIP-1β	?	?	?	→	→
CCL5/RANTES	?	?	?	→	↑
Bone destruction RANKL	?	↓	?	↑	↑
Reference	29	30	33	35	37

pared with wild-type mice[41]. CCL2-deficient mouse-derived peritoneal macrophages expressed CXCL2 mRNA to a greater extent than wild-type mouse-derived ones, when they were stimulated with LPS[42]. Thus, it is plausible that the CCR2-CCL2 axis can have a negative regulatory role in inflammation, particularly CXCL1 and CXCL2 expression. Supporting this notion, Takada et al reported that CCL2 contributes to gut homeostasis by recruiting IL-10-producing macrophages into the lamina propria and that CCL2 deficiency exacerbates dextran sulfate sodium-induced acute colitis together with impaired IL-10-producing regulatory macrophage infiltration[43]. Moreover, aged CCL2- or CCR2-deficient mice developed retinal degenerative changes arising from reduced IL-10 production by monocytes[44]. We also observed that intraarticular expression of IL-10 was lower in IL-1ra-CCR2-doubly-deficient mice than IL-1ra-deficient mice (Fujii H, et al: unpublished observations). Thus, the CCR2-CCL2 axis can regulate the production of IL-10, and the blockade or the lack of the CCR2-CCL2 axis may impair the production of IL-10 and eventually enhance the expression of pro-inflammatory cytokines, IL-1 and TNF, which can enhance the expression of neutrophil-tropic chemokines, CXCL1 and CXCL2.

Evidence is accumulating to indicate that treatment of CCL2 *in vitro* can induce the differentiation of monocytes into osteoclasts[45]. However, bone mineral density was decreased to a greater extent in IL-1ra-CCR2-doubly-deficient mice, compared with IL-1ra-deficient mice[37]. Moreover, osteoclast numbers in synovial tissue and subchondral bone marrow were progressively increased in IL-1ra- and IL-1ra-CCR2-doubly-deficient mice and the increases were more evident in IL-1ra-CCR2-doubly-deficient mice than IL-1ra-deficient mice. Furthermore, the doubly-deficient mice possessed a larger number of monocytes, a precursor of osteoclasts, in bone marrow, than IL-1ra-deficient mice, probably because the lack of CCR2 resulted in the impaired egress of monocytes from bone marrow[46]. Of note is that intraarticular levels of two osteoclastogenic factors, RANKL and a disintegrin and metalloproteinase (ADAM)-8, were increased to greater extents in IL-1ra-CCR2-doubly-deficient mice, than IL-1ra-deficient mice. When CIA was induced in CCR2-deficient mice, RANKL was detected in CD4-positive T lymphocytes in the draining lymph nodes[35]. In contrast, immunofluorescence analysis demonstrated that infiltrating neutrophils, but not T lymphocytes were a major source of RANKL and ADAM-8. Thus, once osteoclastogenic factors are produced by infiltrating neutrophils, these factors may prevail in bone marrow and may induce monocytes in bone marrow to differentiate efficiently into osteoclasts. The generated osteoclasts can spread from bone marrow to synovium and eventually accelerate arthritis progression (Fig.1). However, it still remains unclear how osteoclasts migrate from bone marrow to synovium.

Perspectives

Our present study[37] demonstrated that the lack of CCR2 impaired the egress of monocytes from bone marrow and resulted in the accumulation of monocytes/macrophages in bone marrow (Fig.1). Moreover, in contrast to several *in vitro* studies demonstrating a direct osteoclastogenic activity of CCL2, the lack of its specific receptor, CCR2, actually augments osteoclastogenesis by enhancing infiltration of neutrophils, which are a rich source of osteoclastogenic factors (Fig.1). Similar observations were obtained on experimental dental periapical lesions in mice[47]. However, both studies use CCR2-deficient mice, which lack CCR2 protein from birth. Thus, it is difficult to assess the effects

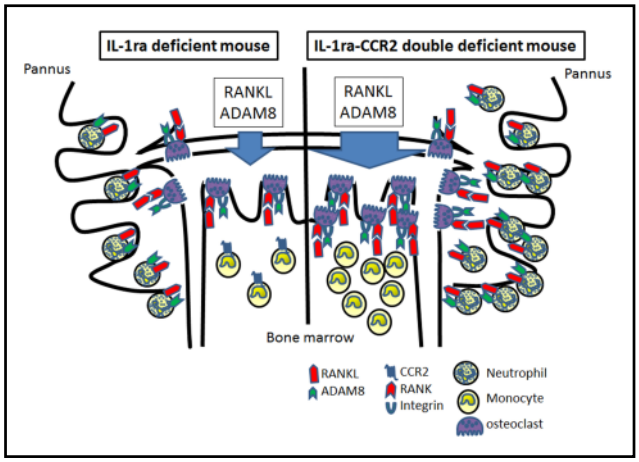

Fig.1 Presumed pathogenic mechanisms of joint lesions in IL-1ra deficient mice and IL-1ra-CCR2 double deficient mice

CCR2 gene deletion impaired the egress of monocytes, a precursor of osteoclasts, from bone marrow and as a consequence, increased the number of monocytes retained in bone marrow. Infiltrating neutrophils expressed two potent osteoclastogenic factors, RANKL and ADAM 8, which can induce monocytes in subchondral bone marrow to efficiently differentiate into osteoclasts. Increased osteoclastogenesis aggravated arthritis as evidenced by enhanced bone erosion and ankylosis.

of CCR2 deficiency on the events after the disease has developed. Nevertheless, our present observations would imply that a CCR2 antagonist may have contradictory effects in a context-dependent manner and therefore, provides a cautionary concern about the clinical application of a CCR2 antagonist, which are being examined in clinical studies targeting multiple sclerosis, systemic lupus erythematosus, arteriosclerosis, and pain[48].

Acknowledgments

We would like to express sincere thanks to Dr. Joost J. Oppenheim (NCI-Frederick, USA) for his invaluable comments on the manuscript. In addition, we express our gratitude to Drs. Yuko Ishida and Toshikazu Kondo (Wakayama Medical University, Wakayama, Japan) for critical advice on the manuscript. We declare no financial or commercial conflict of interest.

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