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S100A4 regulates the Src-tyrosine kinase dependent differentiation of Th17 cells in rheumatoid arthritis

Mikael Brisslert [a], Li Bian [a], Mattias N.D. Svensson [a], Rita F. Santos [b], Ing-Marie Jonsson [a], Igor Barsukov [d], Malin Erlandsson [a], Karin Andersson [a], Alexandre M. Carmo [b,c], Maria I. Bokarewa [a,*]

[a] Department of Rheumatology and Inflammation Research, The Sahlgren's Academy at University of Gothenburg, Sweden
[b] IBMC — Instituto de Biologia Molecular e Celular, University of Porto, Portugal
[c] ICBAS — Instituto de Ciencias Biomedicas Abel Salazar, University of Porto, Portugal
[d] Department of Structural and Chemical Biology, Institute of Integrative Biology, University of Liverpool, United Kingdom

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ABSTRACT

Objectives: To evaluate the role of S100A4, a calcium-binding regulator of nonmuscle myosin assembly, for T-cell responses in rheumatoid arthritis. *Methods*: Arthritis was induced in the methylated bovine serum albumin (mBSA)-immunized mice lacking the entire S100A4 protein (S100A4KO) and in wild-type counterparts treated with short hairpin ribonucleic acid (shRNA)-lentiviral constructs targeting S100A4 (S100A4-shRNA). The severity of arthritis was evaluated morphologically. T-cell subsets were characterized by the expression of master transcription factors, and functionally by proliferation activity and cytokine production. The activity of the Scr-kinases Fyn and Lck was assessed by the autophosphorylation of C-terminal thyrosine and by the phosphorylation of the CD5 cytodomain. The interaction between S100A4 and the CD5 cytodomain was analysed by nuclear magnetic resonance spectrophotometry. *Results*: S100A4-deficient mice (S100A4KO and S100A4-shRNA) had significantly alleviated morphological signs of arthritis and joint damage. Leukocyte infiltrates in the arthritic joints of S100A4-deficient mice accumulated Foxp3$^+$ Treg cells, while the number of RORγt$^+$ and (pTyr705)STAT3$^+$ cells was reduced. S100A4-deficient mice had a limited formation of Th17-cells with low retinoic acid orphan receptor gamma t (RORγt) mRNA and IL17 production in T-cell cultures. S100A4-deficient mice had a low expression and activity of T-cell receptor (TCR) inhibitor CD5 and low (pTyr705)STAT3 (signal transducer and activator of transcription 3), which led to increased (pTyr352)ZAP-70 (theta-chain associated protein kinase of 70 kDa), lymphocyte proliferation and production of IL2. *In vitro* experiments showed that S100A4 directly binds Lck and Fyn and reciprocally regulates their kinase activity towards the CD5 cytodomain. Spectrometry demonstrates an interaction between the CD5 cytodomain and EF2-binding sites of S100A4. *Conclusion*: The present study demonstrates that S100A4 plays an important part in the pathogenesis of arthritis. It controls CD5-dependent differentiation of Th17 cells by regulating the activity of the Src-family kinases Lck and Fyn.

1. Introduction

Rheumatoid arthritis (RA) is a progressive debilitating autoimmune disease, which affects 0.5–1% of the total world population [1–3]. Development of RA is characterized by the inflammatory cell infiltration and severe damage of the affected joints. Mature T-cells are the dominating cellular component of the inflamed and hyperplastic synovia in RA [4]. Intervention in T-cell receptor (TCR) activation by minimizing co-stimulation is proved as an efficient treatment strategy in RA [5] and confirms the central role of T-cells in the pathogenesis of this disease.

Appropriate T-cell responses and the strength of the TCR interaction are linked to cytoskeleton reorganisation, where filamentous actin and nonmuscle myosin II (NMMII) play the major part [6,7]. The activation of TCR recruits effector molecules, where the Src-tyrosine kinases Lck and Fyn play a central part in transmitting a TCR/CD3 signal by phosphorylating ITAMs and activating the tyrosine kinase ZAP-70 [8], and recruits cytoskeletal remodelling factors of the Rho GTPase family to the inner leaflet of the cell membrane. The Rho GTPases are coupled to actin and NMMII, and define the duration of TCR activation [6,7]. The diversion of the NMMII function abolishes the formation of TCR clusters at the outer edge of the T-cell [9] and reduces the activity of Src-tyrosine

Abbreviations: mBSA, methylated bovine serum albumin; WT, wild-type; KO, knock-out; IFNγ, interferon-gamma; IL, interleukin; RORγt, retinoic acid orphan receptor gamma t; RA, rheumatoid arthritis; TNF, tissue necrosis factor; TCR, T-cell receptor; Th, helper T cell; STAT3, signal transducer and activator of transcription 3; ZAP-70, theta-chain associated protein kinase of 70 kDa; RhoA, Ras homolog gene family, member A; IRF4, interferon regulatory factor 4; Fak, focal adhesion kinase; ROCK2, Rho-associated protein kinase 2; MHCII, major histocompatibility complex class II

* Corresponding author at: Department of Rheumatology and Inflammation Research, University of Gothenburg, Guldhedsgatan 10A, Box 480, 405 30 Gothenburg, Sweden. Tel.: +46 31 3424021; fax: +46 31 823925.
E-mail address: maria.bokarewa@rheuma.gu.se (M.I. Bokarewa).

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kinases in T-cells [10]. In the present study, we evaluated the role of S100A4, a regulator of NMMII assembly and an inhibitor of its ATPase activity, in TCR-dependent immune responses and differentiation of T helper subsets.

S100A4 is a small Ca-binding protein known for its metastasis-promoting properties. It is required for normal cell-to-cell interactions and cell motility. In normal cells and tissues, including fibroblasts, macrophages, lymphocytes, and bone marrow-derived haematopoietic progenitors, the increased expression of S100A4 is observed during cell differentiation, and in angiogenesis and organogenesis [11,12]. Over-expression of S100A4 is seen in many types of tumour cells and leads to epithelial–mesenchymal transition and connects S100A4 to its metastasis promoting properties.

The function of S100A4 is best characterized in interaction with cytoskeletal proteins NMMII, F-actin, and tropomyosin. The binding of S100A4 to these proteins occurs in a Ca^{2+}-dependent manner and inhibits the actin-regulated ATPase activity of myosin II [13,14]. The disassembly of myosin filaments occurring as a result of S100A4 binding is viewed as the major impact of S100A4 in cytoskeletal rearrangements, cell polarization, shape changes, and motility [13,15,16]. Recent reports suggest that S100A4 forms a complex with rhotekin, an adaptor molecule to the cytoskeletal remodelling factor RhoA, and is predicted to regulate RhoGTPase-dependent membrane ruffling [17]. S100A4 binds tumour suppressor p53 in the *in vitro* purified system [18] and in cells [19–22]. Binding of S100A4 to p53 occurs in the cell nucleus and initiates its degradation.

The binding of S100A4 to calcium drives conformational changes and permits S100A4 to regulate the activity of its multiple intracellular protein partners placing S100A4 at the crossroad of several intracellular transduction mechanisms. Intracellular S100A4 controls signal transduction through FcγRIIIA by inhibiting the activation of the tyrosine kinase Syk [23]. S100A4 is described as an essential partner of the JAK/STAT signal transduction activating receptors to IL7 in chondrocytes [24] and IL10 in the cells of neuroglia [25]. S100A4 is required for the IL1 receptor dependent activation of an ERK–p38–JNK signalling pathway [26] and for mediating estrogen effects to bone progenitors [27]. S100A4 binds transcription factor Smad3 and enhances TGFγ-mediated effects by promoting cancer invasiveness [28] and autoimmune inflammation [29].

In RA, S100A4 is abundantly expressed in synovial fibroblasts, macrophages and vascular endothelial cells of the inflamed joints and may be measured in synovial fluid and in blood [30,31]. The clinical consequences of the high levels of S100A4 in RA patients are associated with resistant joint inflammation and high skeletal damage [32,33]. The current view on the function of S100A4 is consistent with its extracellular regulation of local inflammation by stimulating the production of matrix metalloproteinases in synovial fibroblasts and in chondrocytes [26,31].

In the present study we show that S100A4 is essential for T-cell maturation and function controlling TCR-dependent immune responses. These results are consistent in two independent *in vivo* models of S100A4-deficiency, the knock-out mice obtained by the germ-line inactivation of the S100A4 gene, and the acute inhibition of the S100A4 gene transcription by specific shRNA-producing constructs. S100A4 directly binds the Src-kinases Lck and Fyn and reciprocally regulates their kinase activity. S100A4-deficiency results in a reduced activity of STAT3 suppressing transcription of RORγt and lineage differentiation of Th17 cells. The immunological events controlled by S100A4 are functionally important for the pathogenesis of arthritis, since the deficiency in S100A4 alleviates experimental arthritis.

2. Materials and methods

2.1. Mice

S100A4 knockout mice (S100A4KO) were generated on an A/Sn background by a germ-line inactivation of the *S100A4* gene as described in [34,35]. The breeding pairs of S100A4KO and congenic WT (A/Sn) mice were kindly provided by Dr. Mariam Grigorian, Institute of Cancer Biology, Copenhagen. Mice were bred at the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg. The mice were housed 8–10 animals/cage with a 12 h light and dark cycle, and fed with standard laboratory chow and water *ad libitum*. All animal experiments are approved by the Animal Experimental Board of the Gothenburg University (permits 2009-88, 319-2011 and 125-2012).

2.2. Arthritis model

Arthritis was induced by the intra-articular injection of 30 μg methylated bovine serum albumin (mBSA, Sigma Aldrich) in the left knee of preimmunised animals as described in [36]. Mice were immunized subcutaneously with mBSA emulsified with the complete Freund's adjuvant (Sigma Aldrich) on day 0 and day 7. The left knee joint was injected with mBSA on day 21 and the morphological evaluation was done on day 28. In total, 33 S100A4KO-mice and 33 WT mice were used in 3 independent experiments. Each experiment contained S100A4KO and WT male littermates, 6–10 mice/group.

2.3. Down-regulation of S100A4 in vivo

Fifteen WT (A/Sn) mice were treated with the bioconstructs containing sequences coding for the S100A4 gene targeting shRNA and a lentiviral vector (Sigma-Aldrich, St. Louis, MO, USA); an additional 7 WT mice received a non-targeting bioconstruct. Mice were treated with 1×10^7 transduction particles/mouse and the successful inhibition of the S100A4 gene transcription was proved by Western blot as described in [37].

2.4. Adoptive transfer

$CD4^+$ T cells were purified on the magnetic beads using the mouse $CD4^+$ T cell isolation kit (StemCell Technology). The purity and viability of the isolated $CD4^+$ T cells were 89% and 98%, respectively. The isolated $CD4^+$ T cells were injected i.v. into the recipient mice (S100A4KO, $n = 7$; and WT mice, $n = 8$). Each mouse obtained 2×10^6 of the CD^+ T cells. S100A4KO mice ($n = 6$) were used as controls. At the same day of $CD4^+$ T cell transfer, mice were subjected to the arthritis model as above and sacrificed on day 28.

2.5. Clinical and histological evaluation of arthritis

The histological evaluation of the mBSA-injected knee joints was done on the paraffin-embedded and haematoxylin and eosin stained sections. The sections were coded and evaluated for signs of inflammation and cartilage/bone destruction. Arthritis was evaluated on an arbitrary scale from 0 to 3 [36]. The representative histological figures of the arthritis scale are shown in Fig. 1A.

2.6. Immunohistochemisty staining

The paraffin-embedded sections of knee joints were subjected to antigen retrieval as described in [38], and blocked with 0.3% H_2O_2, serum solution (Vector laboratories) and Fc-block (BD Pharmingen™). After incubation with rabbit anti-mouse pSTAT3 (Tyr705, AbCam), IL17 (AbCam), RORγt (eBioscience), and Foxp3 (eBioscience) antibodies or rabbit gammaglobulins (Jackson) as a negative control, the specimens were incubated with ImmPRESS anti-rabbit Ig polymer detection reagent and stained using ImmPACT™ AEC (Vector laboratories), and Mayer's haematoxylin (Histolab).