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Small GTPases : emerging targets in rheumatoid arthritis

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Citation for published version (APA):
Ferreira de Abreu, J. R. (2009). Small GTPases : emerging targets in rheumatoid arthritis

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A Rac1 inhibitory peptide suppresses antibody production and paw swelling in the murine collagen-induced arthritis model of rheumatoid arthritis

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Abstract

Introduction

The Rho family GTPase Rac1 regulates cytoskeletal rearrangements crucial for the recruitment, extravasation and activation of leukocytes at sites of inflammation. Rac1 signaling also promotes the activation and survival of lymphocytes and osteoclasts. Therefore, we assessed the ability of a cell-permeable Rac1 carboxy-terminal inhibitory peptide to modulate disease in mice with collagen-induced arthritis (CIA).

Methods

CIA was induced in DBA/1 mice, and either in early or chronic disease, mice were treated three times per week by intraperitoneal injection with control peptide or Rac1 inhibitory peptide. Effects on disease progression were assessed by measurement of paw swelling. Inflammation and joint destruction were examined by histology and radiology. Serum levels of anti-collagen type II antibodies were measured by enzyme-linked immunosorben assay (ELISA). Results were analyzed using unpaired Student's t-tests.

Results

Treatment of mice with Rac1 inhibitory peptide resulted in a decrease in paw swelling in early disease and to a lesser extent in more chronic arthritis. Of interest, while joint destruction was unaffected by Rac1 inhibitory peptide, anti-collagen type II antibody production was significantly diminished in treated mice, both in early and chronic arthritis.

Conclusions

The data suggest that targeting of Rac1 with the Rac1 carboxy-terminal inhibitory peptide may suppress autoantibody production in autoimmune disease. Whether this could translate into clinically meaningful improvement remains to be shown.

Introduction

Rheumatoid arthritis (RA) is marked by de-regulated recruitment, activation, and retention of inflammatory white blood cells in affected joints[1]. Subsequent auto-antibody production, release of cytokines and cell-cell contacts may perpetuate inflammation and lead to joint destruction through activation of stromal fibroblast-like synoviocytes (FLS) and osteoclasts[2]. Many of the cellular processes required for perpetuation of inflammation and joint destruction in RA are regulated by Rac GTPases, members of the Rho-like family of small GTPase signaling proteins[3].
Rac1 is ubiquitously expressed in mammalian tissues, while expression of Rac2 is limited to cells of hematopoietic lineage[4;5]. Rac GTPases are activated by a broad array of extracellular stimuli relevant to RA, including chemokines, lymphocyte antigen receptor ligation, inflammatory cytokines, and cell-cell adhesion[6-11]. Following activation, Rac proteins initiate multiple signaling pathways that regulate cytoskeletal rearrangements, kinase cascades needed for gene transcription, and assembly of the NADPH oxidase[6-12]. Transfection of active and dominant-negative mutants of Rac1, as well as genetic studies, have demonstrated that lymphocytes and neutrophils require Rac1 signaling for efficient polarized chemotactic responses and trafficking in vivo[13-19]. Although macrophages do not require Rac1 and Rac2 function for chemotactic responses, macrophage invasion of tissue is dependent upon Rac1 and Rac2[20]. Rac signaling is also important for productive interactions between lymphocytes and antigen presenting cells (APCs). After antigen recognition by T-cells, ezrin-radixin-moesin (ERM) proteins are dephosphorylated through a Rac1-dependent pathway, favoring relaxation of the cytoskeleton and subsequently promoting T-cell −APC conjugate formation[21]. Reciprocally, Rac activity in dendritic cells is required for effective antigen presentation to T-cells and subsequent T-cell priming[22]. Antigen receptor-dependent activation of Rac signaling also stimulates activation of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and NF-κB signaling pathways important for lymphocyte activation, proliferation, and survival[8-10]. Many of these downstream signaling pathways are now being explored as potential therapeutic targets in RA[23]. Rac proteins also serve additional important functions in cells of myeloid lineage which contribute to inflammation and joint destruction in RA. Oxidative bursts of macrophages and neutrophils rely upon Rac1-dependent assembly of the NADPH oxidase machinery[12]. Additionally, in vitro studies of osteoclasts transfected with plasmid encoding dominant-negative Rac, and in vivo studies in Rac-deficient mice have identified essential but redundant roles for Rac1 and Rac2 proteins in osteoclastogenesis, osteoclast motility, and bone resorption[24;25].

Together, these studies indicate that therapeutic strategies targeting Rac1 function may be of clinical benefit in RA. However, pre-clinical assessment of Rac1 inhibition has been hampered by a lack of compounds specifically targeting Rac1, and limited analyses of Rac1 in animal models of arthritis, a consequence of early findings demonstrating that genetic deletion of Rac1 in mice results in early embryonic lethality[26]. NSC23766, a pharmacological compound which inhibits all Rac GTPases, suppresses RA FLS proliferation and invasiveness in vitro, effects mimicked by siRNA silencing of Rac1 expression in these cells[27]. This may indicate that specific inhibition of Rac1 may be therapeutically beneficial in RA. However, mice in which Rac1 has been conditionally deleted in mature neutrophils and macrophages, on a Rac2-deficient background, show a complex phenotype in a Chlamydia-induced infection model of arthritis[28]. In these animals, Rac1 has a bimodal effect on disease progression. In the acute phase, Rac1-deficiency delays recruitment and activation of inflammatory neutrophils in the joint, while in the chronic phase, disease is exacerbated due to an inability of neutrophils to clear the pathogen. In this study, we targeted Rac1 in mice with CIA, using a Rac1-specific cell-permeable carboxy-terminal inhibitory peptide which we have previously shown to block Rac1 function in human lymphocytes, endothelial cells and epithelial cells[11;29;30].

Materials and methods

Animals

Male DBA/1 mice were purchased from Harlan (Horst, The Netherlands), housed under conventional conditions at the animal facility of the Academic Medical Center (Amsterdam, The Netherlands), and fed ad libitum. The animal ethical committee of the Academic Medical Center approved all experiments.

Peptide synthesis

For this study, peptides encoding a protein transduction domain[31] alone (indicated as control, Ctrl throughout the manuscript) or fused to the carboxy-terminal domain of Rac1, excluding the Rac1 CAAX box (indicated as Rac1 throughout the manuscript), were synthesized using N-(9-fluorenyl)methoxycarbonyl (fMoc) solid phase

chemistry[30]. Peptide synthesis was performed using a Syro II (MultiSynTec GmbH, Bochum, Germany).

T-cell isolation

Murine spleens were crushed through a 40-μm cell strainer (BD Pharmingen, Franklin Lakes, NJ) to obtain single cell suspensions. Erythrocytes were lysed with ice-cold isotonic NH4Cl solution (155 mM NH4Cl, 10 mM KHCO3, and 100 mM EDTA, pH 7.4). To purify T-cells, splenic cell suspensions were incubated with anti-murine CD4 and CD8 antibody-coated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min at 4°C and positively selected by magnetic separation with MACS. Purified T-cells were >90% CD3+, as analyzed by flow cytometry.

Actin polymerization assays

Actin polymerization assays were performed as previously described[32]. Briefly, purified T-cells were pre-incubated for 15 minutes in medium containing 200 μg/ml Ctrl or Rac1 peptide. T-cells were then exposed to 100 ng/ml SDF-1, and at the indicated time points 100 μl aliquots of cell suspensions were transferred to an equivalent volume of fixation solution (Intraprep Fixation Reagent, Coulter Immunotech, Marseille, France). After 15 minutes, cells were washed in 0.5% bovine serum albumin/phosphate-buffered saline (PBS) and resuspended in 100 μl of permeabilization reagent (Coulter Immunotech) for 5 minutes. Cells were stained for 20 minutes with 1 unit/ml Alexa 488 Phalloidin (Invitrogen, Molecular Probes, Eugene, OR) to visualize F-actin. The mean fluorescence intensity (MFI) of polymerized actin was measured by FACS (BD Biosciences), and the fold increase in actin polymerization was calculated by dividing the MFI generated at a particular time point by the MFI at t = 0 of that particular condition.

Induction and assessment of CIA

Bovine collagen type II (bCII) (2 mg/ml in 0.05 M acetic acid, Chondrex, Inc., Redmond, WA) was mixed with complete Freund's adjuvant (CFA) (2 mg/ml of Mycobacterium tuberculosis, Chondrex, Inc.) and injected intradermally on day 0 at the base of the tail with 100 μl of emulsion into 8- to 11-week old mice. On day 21, mice received an intraperitoneal (ip) booster injection with 100 μg of bCII in PBS. To in-

vestigate the treatment efficacy of Rac1 peptide at disease onset, mice were treated at day 20 with either 2mg, 1mg or 0.5mg of Ctrl or Rac1 peptide, three times weekly until sacrifice. Alternatively, to explore the effect of Rac1 peptide treatment in chronic disease, the animals were randomly assigned at day 29 to one of two groups, and treated ip with 4 mg Ctrl, or Rac1 peptide. Treatments were continued three times weekly until sacrifice at day 39. The severity of arthritis was assessed in a blinded manner, using a semi-quantitative scoring system (0 to 4): 0, normal; 1, redness and/or swelling in one joint; 2, redness and/or swelling in more than one joint; 3, redness and/or swelling in the entire paw; and 4, deformity and/or ankylosis. Hind paw ankle joint thickness was measured using a dial caliper (POCO 2T 0- to 10-mm test gauge; Kroeplin Längenmesstechnik, Schlüchtern, Germany). Experiments were performed using 8–16 mice per group.

Histologic analysis

Hind paws were fixed in 10% buffered formalin for 48 hours and decalcified in 15% ethylenediaminetetraacetic acid (EDTA). The paws were then embedded in paraffin, and 5-μm saggital serial sections of whole hind paws were cut. Tissue sections were stained with hematoxylin and eosin. Inflammation was graded on a scale from 0 (no inflammation) to 3 (severely inflamed joint) based on infiltration of the synovium by inflammatory cells. Cartilage erosion was scored using a semi-quantitative scoring system from 0 (no erosions) to 3 (extended erosions). The tissue was examined by microscopic evaluation in a blinded manner by two independent observers (JA and MV).

Radiological analysis

Hind paws were used for radiographic evaluation. Two observers without knowledge of the treatment groups scored the X-rays. Joint destruction was scored on a scale from 0 to 4: 0, no damage; 1, minor bone destruction observed in one enlightened spot; 2, moderate changes, two to four spots in one area; 3, severe erosions afflicting the joint; and 4, complete destruction of the joints.

Determination of anti-collagen antibodies by ELISA

Maxisorb 96-well plates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml of bCII in 0.1 M sodium carbonate buffer (pH 9.7) overnight at 4°C. After blocking for 1 h with 2% milk in PBS at room temperature, sera were added in serial dilutions in 2% milk/PBS, and incubated overnight at 4°C. Plates were subsequently washed and incubated with 1 µg/ml biotinylated rat anti-mouse immunoglobulin (Ig) (Southern Biotechnology Associates, Birmingham, AL) of the indicated isotype in 2% milk/PBS for 1 h at room temperature. After washing, plates were incubated with streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch, Newmarket, Suffolk, UK) for 1h at room temperature, washed, and developed with p-nitrophenyl phosphate substrate (Sigma-Aldrich, St Louis, MO). The reaction was stopped with 2M H_2SO_4, and optical density (OD) at 415 nm was measured.
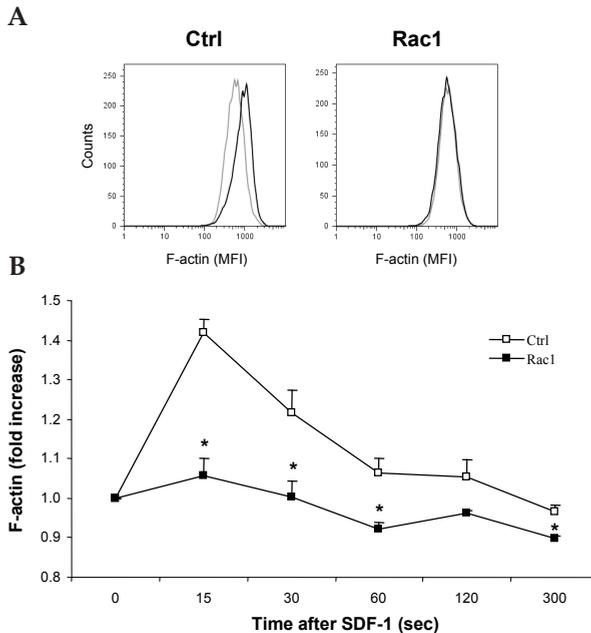

Figure 1. The Rac1 carboxy-terminal peptide efficiently blocks actin polymerization in murine cells. *Representative histograms of F-actin staining in T-cells isolated from mice spleens that were exposed for 15 min to 200 ug/ml of Ctrl or Rac1 peptide followed by SDF-1 stimulation (a). Fold increase in F-actin (b). Data are depicted as mean fold increase of F-actin ± standard error of the mean (SEM) (n=3); *P < 0.05.*

Statistical analysis

To evaluate the effects of different treatments, we determined the change in paw swelling scores (delta) of each mouse from the start of treatment until the end of the experiment. Areas under the curve (AUC) were calculated for the delta paw swelling. The significance of the differences between the means of delta paw swelling, radiological, and histologic scores between groups was determined by using the unpaired Student's t-test. P values ≤ 0.05 were considered statistically significant.

Results

The Rac1 inhibitory peptide blocks murine T-cell actin polymerization

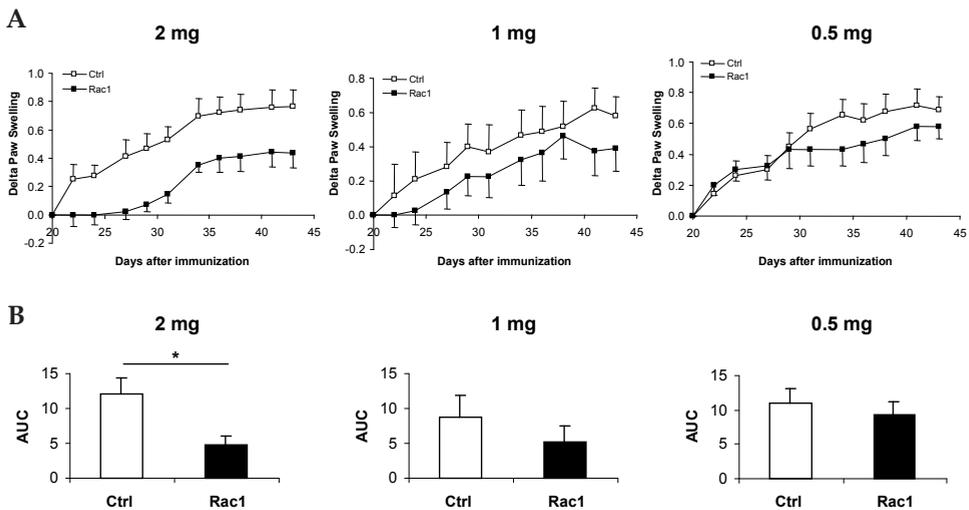The Rac1 peptide is able to block endogenous Rac1 signaling within minutes by

Figure 2. Reduced paw swelling after treatment with 2mg of Rac1 carboxy-terminal peptide.
Mice were treated with 2 mg, 1 mg or 0.5 mg Ctrl (□) or Rac1 (■) peptide at the indicated time points. Paw swelling and inflammation of the four limbs were determined for each mouse. Delta hind paw ankle joint swelling was calculated by subtracting the paw diameter on the day of initiation of treatment from the measured diameter (a). Values are depicted as mean ± SEM (n=8). Area under the curve (AUC) was calculated for the delta paw swelling in each mouse (b). Values are depicted as mean AUC ± SEM. *P < 0.01.

competing with Rac1 effector proteins, and in vitro has been demonstrated to possess a potent capacity to block actin polymerization and migration of human cells stimulated with SDF-1[11;29;30]. The biological activity of the peptide batches used for in vivo experiments, and their ability to influence murine cellular responses, was first examined in an actin polymerization assay using murine splenic T-cells. Pre-treatment of murine T-cells with Rac1 peptide, but not Ctrl peptide, completely blocked actin polymerization following SDF-1 stimulation, as measured by increases in T cell F-actin content (Figures 1A and 1B). This indicated that the Rac1 peptide was effective in blocking Rac1 signaling not only in human cells, but murine cells as well.

Rac1 inhibitory peptide treatment reduces paw swelling and anti-bCII antibody production in early arthritis

After confirming the in vitro efficiency of the Rac1 peptide in inhibiting murine Rac1
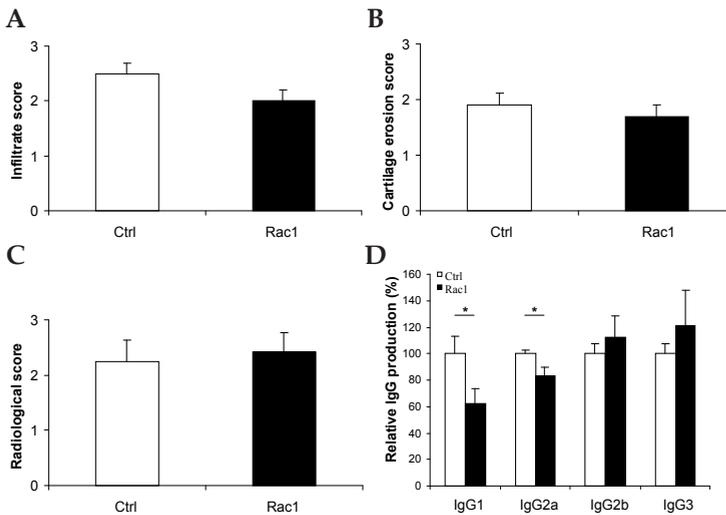

Figure 3. Treatment with the Rac1 carboxy-terminal peptide at onset of disease reduces anti-bovine collagen IgG production but does not protect against joint destruction. *Mice were treated with Ctrl (white bars) or Rac1 (black bars) peptide. Sections from mice paws were stained with haematoxylin and eosin (n=8). X-rays of hind paws were analyzed for bone damage (n=8). Cellular infiltration scores (a). Cartilage erosion scores (b). X-ray scores showing no differences in bone damage between groups (c). Values are depicted as mean ± SEM. Serum from mice that started treatment at day 20 (n=8) with Ctrl (white bars) or Rac1 (black bars) peptide were collected and the levels of specific anti-collagen IgG detected (d). IgG levels in the serum of Ctrl-treated mice were set to 100% and the levels obtained in the serum of Rac1 -treated mice were then calculated relative to Ctrl, and expressed as mean ± SEM. Represented values were calculated within linear regions of the serum dilution curve. *P ≤ 0.05.*
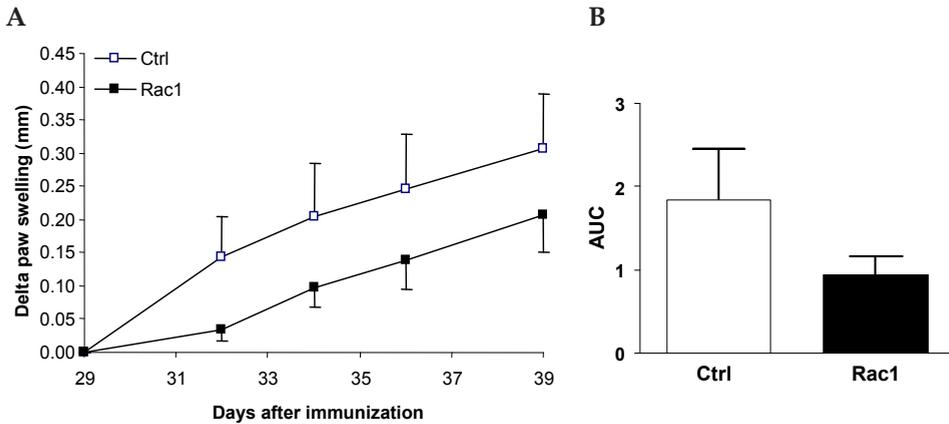
A

B

Figure 4. Effect of Rac1 carboxy-terminal peptide treatment of mice with chronic arthritis on paw swelling. *Hind paw swelling was monitored during the course of disease using calipers. Delta hind paw ankle joint swelling of mice treated at day 29, three times weekly, with 4 mg Ctrl or Rac1 peptide (a). Values are depicted as mean ± SEM and are representative of two independent experiments (n= 16 per group). AUC calculated as in Figure 2b (b).*

signaling, we examined the in vivo potential of this peptide when mice are treated at the onset of disease. One day before the booster, at day 20, we started treatment of the animals with 2 mg, 1 mg or 0.5 mg Ctrl or Rac peptide, three times weekly, until sacrificing. Treatment of animals with Rac1 peptide at all doses failed to influence disease incidence, clinical scores of disease severity or animal weight (data not shown) but animals treated with 2 mg of Rac1 peptide showed a highly significant decrease in paw swelling when compared to treatment with Ctrl peptide (61% reduction, P = 0.009) (Figures 2A and 2B). The effect was dose-dependent; treatment with lower doses of Rac1 peptide showed a trend towards improvement, which did not reach statistical significance (Figures 2A and 2B).

We next examined the influence of Rac1 peptide on synovial inflammation, cartilage degradation, and bone destruction. Quantification of synovial inflammation revealed a minor decrease in cellularity, which did not reach statistical significance, possibly due to the relatively small number of mice (Figure 3A). Treatment with Rac1 peptide did not protect against joint destruction (Figures 3B and 3C).

Finally, we examined the influence of Rac1 peptide on anti-bCII antibody production. We collected sera from mice at the time of sacrifice and measured specific anti-bCII antibody levels by ELISA. Mice treated with Rac1 peptide showed a significant
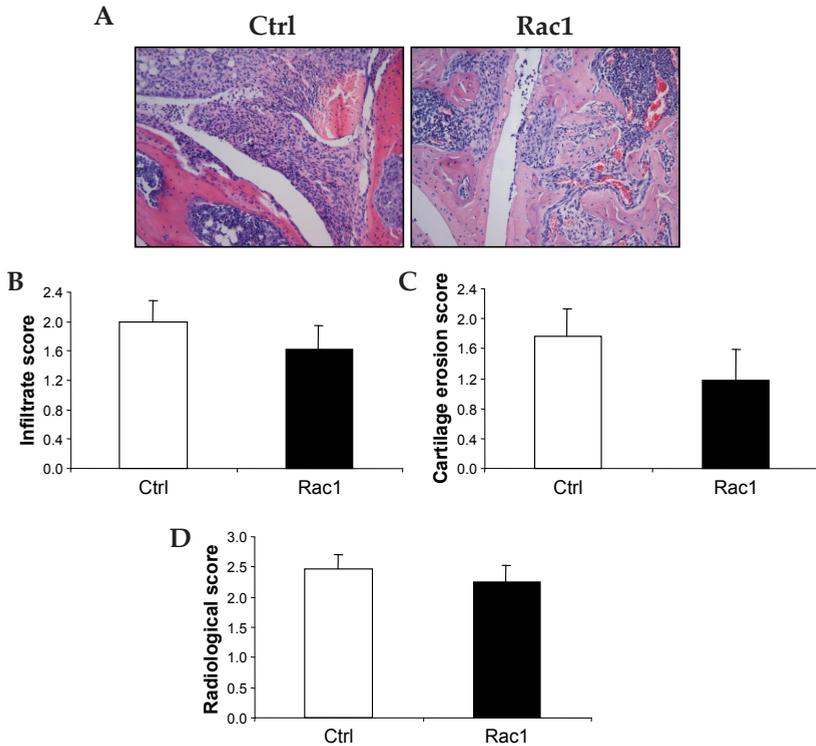
Figure 5. Rac1 carboxy-terminal peptide treatment of mice with chronic arthritis does not protect against joint destruction. *Mice were treated with Ctrl (white bars) or Rac1 peptide (black bars). Paraffin-embedded sections of the hind paws were stained with haematoxylin and eosin and analyzed for synovial inflammation and cartilage destruction. X-rays were analyzed for bone damage (n = 16 per group). Representative pictures of joints showing extensive cellular infiltration and cartilage erosions (a). Cellular infiltration scores (b). Cartilage erosion scores (c). X-rays from mice paws were taken and scored for bone damage (d). Values are depicted as mean ± SEM.*

reduction in the serum levels of anti-bCII IgG1 (Ctrl 100% ± 12.9; Rac1 62.1% ± 11.8; P < 0.05) and IgG2a (Ctrl 100% ± 2.7; Rac1 83.3% ± 6.8; P = 0.05) antibodies compared to mice treated with Ctrl peptide (Figure 3D).

Treatment of chronic CIA with Rac1 peptide reduces anti-bCII antibody production

We next investigated the effect of Rac1 peptide treatment of mice with chronic

arthritis. For this we performed an independent CIA experiment wherein 29 days after the initial immunization, mice having clinical signs of arthritis were randomly assigned to one of two groups. Mice within groups were treated with 4 mg of Ctrl or 4 mg of Rac1 peptide, three times weekly, until sacrificing. Administration of Rac1 peptide had no influence on clinical disease severity (data not shown). However, we observed a clear trend towards reduced paw swelling in mice treated with Rac1 peptide (Figure 4A and 4B) in two independent experiments, although the differences did not reach statistical significance (experiment 1: 49% reduction, $P = 0.528$; experiment 2: 22% reduction, $P = 0.193$) (Figure 4B).

We also analyzed the effects of the Rac1 peptide on synovial inflammation, cartilage degradation, and bone destruction. Consistent with the trend towards reduced paw swelling, there was a trend towards decreased histologic signs of inflammation and cartilage destruction, which did not reach statistical significance (Figure 5A, 5B and 5C). Analysis of X-rays taken from the mice paws revealed that Rac1 peptide treatment did not protect against erosive disease (Figure 5D). However, Rac1 peptide treatment of mice with chronic arthritis resulted in a significant reduction in the serum levels of anti-bCII IgG2a (Ctrl 100% ± 5.6; Rac1 81.6% ± 6.5; $P = 0.05$) and IgG2b (Ctrl 100% ± 10.9; Rac1 56.0% ± 6.7; $P < 0.005$), whereas no differences were observed for IgG1 or IgG3 (Figure 6).
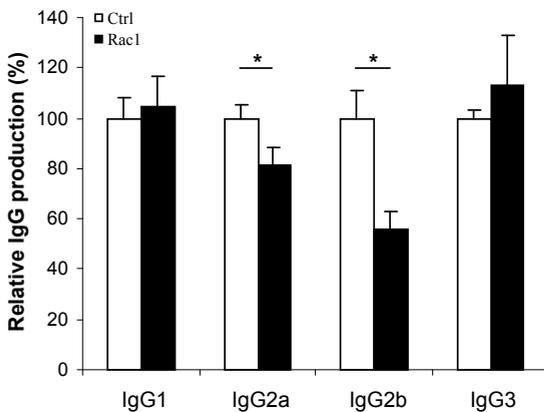

Figure 6. Rac1 carboxy-terminal peptide treatment of mice with chronic arthritis reduces anti-collagen IgG production. *Serum from mice that started treatment at day 29 (n=7) with Ctrl (white bars) or Rac1 peptide (black bars) were collected and the levels of specific anti-collagen IgG detected. IgG levels in the serum of Ctrl-treated mice were set to 100% and the levels obtained in the serum of Rac1 peptide-treated mice were then calculated relative to Ctrl, and expressed as mean ± SEM. Represented values were calculated within linear regions of the serum dilution curve. *P ≤ 0.05.*

Discussion

In this report we provide evidence that the inhibitory Rac1 carboxy-terminal peptide suppresses anti-collagen antibody production in mice with CIA, associated with reduced paw swelling. We have found that the administration of Rac1 peptide in vivo, either as an early treatment or as treatment of mice with more chronic arthritis, results in a significant reduction of circulating levels of anti-bCII IgG1 and IgG2a or IgG2a and IgG2b antibodies, respectively, dependent on the stage of the disease. In both early and chronic arthritis, there was an effect on IgG2a levels. In many murine in vivo model systems, IgG2a and IgG2b antibodies display greater pro-inflammatory properties than IgG1 and IgG3[33]. Genetic studies indicate that the suppressive effect of Rac1 peptide on anti-collagen antibody production observed in our studies is unlikely to be due to direct effects on B-cell trafficking or activation. Rac1, together with Rac2, is critical in transducing B-cell receptor signals required for both survival and efficient cell cycle entry[9;34]. Rac2 deletion in mice results in decreased B-cell maturation and T-cell -independent antigen responses[13;14]. In contrast, conditional deletion of Rac1 in the B-cell compartment has no observable effect on B-cell maturation or function unless Rac2 is simultaneously deleted[34]. Similarly redundant but critical roles for Rac1 and Rac2 are observed in T-cells[17]. In addition, antibody production is critically dependent on efficient T-cell priming. Deletion of Rac1, but not Rac2, inhibits migration of $CD8\alpha^+$ dendritic cells (DCs) to secondary lymphoid organs[22]. Moreover, Rac-deficient DCs fail to establish stable contacts with naïve T-cells, leading to suboptimal T-cell priming[22]. DCs from mindin^{-/-} mice, which have reduced expression of Rac1 and Rac2, also have impaired priming capacity due to inefficient engagement with T-cells, in turn leading to defective humoral responses to T-cell -dependent antigens in these mice[35]. This may suggest that Rac1 peptide suppresses anti-collagen antibody production in CIA via inhibition of lymphocyte interactions with antigen presenting cells.

Surprisingly, we observed little if any effect of Rac1 peptide on cartilage and joint destruction in murine arthritis, although a significant decrease in paw swelling was observed in mice when treated at the onset of disease, and a reproducible trend towards reduced paw swelling was noted in mice treated with Rac1 peptide at a more chronic phase of disease. There was only a limited effect of Rac1 peptide treatment on synovial cellularity, which might be in part due to the often redundant roles of Rac1 and Rac2 in lymphocytes and myeloid lineage cells, and the high specificity of the Rac1 peptide for interfering with signaling from Rac1 but not other Rho family GTPases[30]. Murine neutrophils express both Rac1 and Rac2, and genetic deletion of

each GTPase has revealed their important but redundant contributions to neutrophil chemotactic responses in vitro and in vivo[16;19]. Rac1-deficient murine neutrophils retain chemokinetic responses, but are unable to orient and migrate towards chemokine gradients. In contrast, Rac2 is required for efficient neutrophil migration[19]. Although similar direct comparative analyses have not been performed on B and T -cells in these mice, initial studies indicate that lymphocyte trafficking is regulated primarily by Rac2[13]. Lack of effect of Rac1 peptide on joint destruction in CIA may reflect a redundant role for Rac2 in supporting osteoclastogenesis[24;25].

Decreases in paw swelling after treatment with Rac1 peptide which we observed might be due to effects on the formation of edema. Experiments conducted in vitro have demonstrated that exposure of human endothelial cells to reactive oxygen species, or engagement of the integrin ligand VCAM, leads to Rac1 dependent loss of cadherin-mediated endothelial cell-cell adhesion. In the presence of the Rac1 peptide, endothelial cell-cell adhesion is maintained (data not shown)[11;36]. A role for Rac1 in maintaining vascular endothelial integrity in vivo is also indirectly suggested in studies of c-Jun knockout mice, where inhibition of c-Jun, a downstream target of Rac signaling, suppresses edema, paw swelling and inflammation in an experimental model of arthritis[37]. Our results suggest that future studies should also consider the potential contributions of Rac2, independently or in conjunction with Rac1, to pathology in CIA. Structure-based studies have recently led to the development of small molecular weight compounds which can specifically prevent interaction of Rac1 and Rac2 with activating guanine nucleotide exchange factors[27;38]. These compounds can block RA FLS growth and matrix invasion in vitro, although their efficacy in the treatment of arthritis in vivo remains to be established.

Conclusions

We demonstrate that a cell-permeable inhibitory Rac1 carboxy-terminal peptide can reduce paw swelling and antibody production during murine experimental arthritis. Conceivably, Rac1 peptide treatment could augment the pharmacologic activity toward B-lineage cells of other immunosuppressive therapies, like rituximab or atacicept, which may theoretically increase therapeutic activity. An alternative approach that might perhaps result in a beneficial effect both on clinical signs and symptoms, as well as joint destruction could be the suppression of Rac signaling in RA by compounds targeting both Rac1 and Rac2 signaling. The present study supports the rationale for future studies exploring these approaches.

Acknowledgments

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