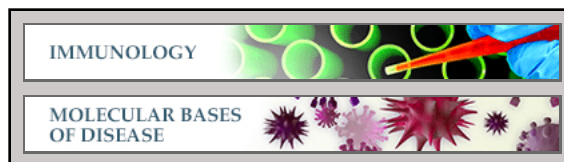


**Immunology:**

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# Ras Guanine Nucleotide-releasing Protein-4 (RasGRP4) Involvement in Experimental Arthritis and Colitis\*

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**Background:** Mast cells express RasGRP4, which is a guanine nucleotide exchange factor and diacylglycerol/phorbol ester receptor.

**Results:** A RasGRP4-null C57BL/6 mouse line was created to evaluate the importance of this signaling protein.

**Conclusion:** RasGRP4 participates in experimental colitis and arthritis.

**Significance:** The inactivation of RasGRP4 might be of therapeutic benefit in some inflammatory diseases.

RasGRP4 (Ras guanine nucleotide-releasing protein-4) is an intracellular, calcium-regulated guanine nucleotide exchange factor and diacylglycerol/phorbol ester receptor expressed in mast cells (MCs) and their progenitors. To study the function of this signaling protein in inflammatory disorders, a homologous recombination approach was used to create a RasGRP4-null C57BL/6 mouse line. The resulting transgenic animals had normal numbers of MCs in their tissues that histochemically and morphologically resembled those in WT C57BL/6 mice. MCs could also be generated from RasGRP4-null mice by culturing their bone marrow cells in IL-3-enriched conditioned medium. Despite these data, the levels of the transcripts that encode the proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  were reduced in phorbol 12-myristate 13-acetate-treated MCs developed from RasGRP4-null mice. Although inflammation was not diminished in a *Dermatophagoides farinae*-dependent model of allergic airway disease, dextran sodium sulfate-induced colitis was significantly reduced in RasGRP4-null mice relative to similarly treated WT mice. Furthermore, experimental arthritis could not be induced in RasGRP4-null mice that had received K/BxN mouse serum. The latter findings raise the possibility that the pharmacologic inactivation of this intracellular signaling protein might be an effective treatment for arthritis or inflammatory bowel disease.

Mast cells (MCs)<sup>2</sup> have prominent adverse roles in numerous inflammatory diseases. Thus, a major effort is under way to identify the factors that regulate the development of mouse and human MCs from their hematopoietic progenitors. Less is known about the intracellular signaling proteins downstream of those surface receptors that regulate the differentiation, maturation, and function of MCs from their hematopoietic progenitors. Mouse bone marrow-derived MCs (mBMMCs) differentiated *in vitro* with IL-3 (1) are immature non-transformed cells that develop into phenotypically different populations of tissue MCs when adoptively transferred into MC-deficient *W/W'* mice (2). Prior to the release of the first drafts of the mouse and human genomes, we isolated a 2.1-kb cDNA from mBMMCs that encodes a 75-kDa protein that had not been identified in any species at the gene, mRNA, or protein level (3). It was designated RasGRP4 (Ras guanine nucleotide-releasing protein-4) because of its ~50% amino acid similarities to mouse and human RasGRP1, RasGRP2, and RasGRP3.

The ~19-kb mouse *RasGRP4* gene was isolated and mapped to chromosome 7B1 (4). Its human (3) and rat (5) orthologs were then isolated using homology-based cloning approaches. Reuther *et al.* (6) also isolated a RasGRP4 cDNA from a patient with acute myeloid leukemia. RT-quantitative PCR, RNA blot, and immunohistochemical data revealed that RasGRP4 is expressed in mature mouse and human MCs and their circulating progenitors. At the cellular level, the *RasGRP4* transcript was detected in all examined mouse MC lines. Based on the accumulated data, RasGRP4 is expressed relatively early in developing MCs, and this signaling protein continues to be expressed in MCs as they differentiate and undergo granule maturation in tissues. Because human mononuclear phagocytes and MCs originate from a common CD34<sup>+</sup> progenitor (7), the failure to detect immunoreactive RasGRP4 protein in macrophages suggests that this intracellular signaling protein

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<sup>2</sup> The abbreviations used are: MC, mast cell; mBMMC, mouse bone marrow-derived MC; PMA, phorbol 12-myristate 13-acetate; ES, embryonic stem; mMCP, mouse MC protease; BAL, bronchoalveolar lavage; DSS, dextran sodium sulfate.

## RasGRP4-dependent Arthritis and Colitis

ceases to be expressed when circulating multipotential CD34<sup>+</sup> progenitors become mature macrophages in tissues.

RasGRP4 is a guanine nucleotide exchange factor that can activate H-Ras efficiently (3, 4, 6). Immunogold ultrastructural studies of the *in vivo* differentiated MCs in the V3 mastocytosis mouse revealed that RasGRP4 tends to reside either in the cytoplasm or on the cytosolic side of the plasma membrane (4). The latter data imply that RasGRP4 participates in early signaling events at the surface of the MC with an undefined receptor. Subcellular fractionation studies of RasGRP4-expressing transfectants (3) confirmed that RasGRP4 is often located in the plasma membrane even though this guanine exchange factor lacks a membrane-spanning hydrophobic domain or a myristoylation/palmitoylation modification site. We therefore concluded that an intracellular factor or post-translational modification event regulates the movement of RasGRP4 from the cytoplasm to the inner leaflet of the plasma membrane.

Activation of MCs often results in the generation of diacylglycerol, and the transient production of this intracellular lipid has been linked to various signaling events in MCs. RasGRP4 possesses a 50-mer domain in its C-terminal half that closely resembles a 50-mer domain in PKC that recognizes phorbol esters and diacylglycerol. Phorbol 12-myristate 13-acetate (PMA) treatment of fibroblast transfectants that were forced to express RasGRP4 resulted in morphologic changes in the resulting cells (3, 4, 6), and site-directed mutagenesis/expression studies confirmed that residues 537–590 in mouse RasGRP4 bind PMA (4). Finally, rescue of a RasGRP4-defective variant of the HMC-1 MC line (3) with a construct that encodes GFP-labeled RasGRP4 confirmed the functional importance of the C-terminal PMA/diacylglycerol-binding domain in the protein (8).

We now describe the creation of a RasGRP4-null C57BL/6 mouse line. Our finding that experimental arthritis and colitis were significantly reduced in these transgenic mice relative to WT controls reveals for the first time a prominent role for this signaling protein in certain inflammatory disorders.

### EXPERIMENTAL PROCEDURES

**Targeting of RasGRP4 Locus in Mouse Embryonic Stem (ES) Cells and Generation of the RasGRP4<sup>-/-</sup> C57BL/6 Mouse Strain**—The *RasGRP4* gene (GenBank<sup>TM</sup> accession number 233046) (4) resides on mouse chromosome 7B1 downstream of the *Ryr1* gene and upstream of the *Spred3* gene. Using genomic DNA from C57BL/6 mice and oligonucleotides based on the genomic sequence of the chromosomal locus at GenBank<sup>TM</sup> accession number NC\_000073, a PCR approach was carried out to obtain the *RasGRP4*-specific nucleotide sequences for the targeting vector noted in Fig. 1A. Employing the translation initiation site of the *RasGRP4* transcript as a reference point (defined here as residue +1), a 2498-bp 5'-homology arm (nucleotides –2497 to 0) was cloned into the BglII-HindIII sites of the pKO-Scrambler NTK-1907 plasmid (Stratagene) downstream of the phosphoglucokinase promoter-driven neomycin resistance gene (PGK-*Neo*). A 3508-bp 3'-homology arm (corresponding to nucleotides +10916 to +14424) was then cloned into the SacII-SalI sites upstream of PGK-*Neo*. The herpes simplex virus thymidine kinase cDNA

driven by a mutant polyomavirus enhancer (MC1-TK) was used for negative selection. The linearized targeting vector was electroporated into C57BL/6 ES cells that were grown in the presence of neomycin and gancyclovir. Surviving ES cell clones that underwent homologous recombination were identified by DNA blot analysis of XmnI-digested genomic DNA hybridized with a radiolabeled 0.9-kb probe corresponding to a nucleotide sequence outside the targeting construct just downstream of the 3'-homology arm.

Selected ES cell clones were microinjected into C57BL/6J-*Tyr<sup>c-2j</sup>/J* blastocysts, which were then implanted into pseudo-pregnant CD-1 foster mothers. The resulting male chimeric mice were crossed with C57BL/6J-*Tyr<sup>c-2j</sup>/J* females, and non-albino pups from this animal cross were screened for germ line transmission of the mutant *RasGRP4* allele. Genomic DNA was isolated from proteinase K-digested mouse tails. RasGRP4<sup>+/+</sup>, RasGRP4<sup>+/-</sup>, and RasGRP4<sup>-/-</sup> mice were identified using the PCR genotyping approaches noted in Fig. 1. We used a primer set (P1/P2) flanking the 3'-homology arm to confirm homologous recombination while backcrossing with WT C57BL/6 mice for nine generations to dilute the effects of possible non-homologous recombination and other genomic alterations. After that, we used the P3 (5'-CGGTAGTGGGCATAGTTGTTG-3')/P4 (5'-TGCCTCTTTGTGTGTTTCATATTC-3')/P5 (5'-TTCCATTTGTCACGTCCTGCAC-3') 3-mer primer set at a ratio of 1.0:0.75:0.25 in a PCR with an initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 40 s and a final extension time of 5 min.

The resulting RasGRP4-null C57BL/6 mice were housed in isolated barrier animal facilities of The University of Texas MD Anderson Cancer Center and Taconic (Germantown, NY). Mice from the latter colony were transported into the animal facility at the Brigham and Women's Hospital/Dana-Farber Cancer Institute, where they were allowed to acclimate for at least 1 week prior to any experiment. Mice were maintained on LabDiet 5001 (Purina, St. Louis, MO) and water. All animal studies were carried out using protocols approved by our institutions' Institutional Animal Care and Use Committees.

**Harvesting and Processing of Peritoneal MCs**—After euthanasia with CO<sub>2</sub>, 10 ml of PBS was injected into the peritoneal cavity of each mouse and then aspirated. The resulting lavage fluid was centrifuged at 450 × *g* for 5 min, and the cell pellet obtained was resuspended in 0.5 ml of PBS. For cell analysis, the number of nucleated cells in 10 μl of each lavage fluid was counted using a Neubauer chamber. For differential counts, 100-μl cytospin samples were Wright-Giemsa-stained. For flow cytometry, 5 × 10<sup>4</sup> cells were incubated with 200 ng of phycoerythrin-Cy7-conjugated anti-mouse Kit/CD117 and 200 ng of Alexa Fluor 647-conjugated anti-mouse FcεRIα (eBioscience) antibodies in 100 μl of PBS for 30 min at 4 °C. The labeled cells were washed twice with PBS, resuspended in 0.5 ml of PBS, and analyzed in a four-laser LSR II flow cytometer (BD Biosciences). The number of CD117<sup>+</sup>/FcεRIα<sup>+</sup> cells and their mean fluorescence intensity at the emission ranges of the fluorophores were recorded because the obtained data should be proportional to the amount of the two receptors expressed on the surfaces of the MCs.



For electron microscopy,  $5 \times 10^4$  cells were fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate (pH 7.2) for 2 h and then in 1% osmium tetroxide for 1 h. After washing in double-distilled  $H_2O$ , the cells were pelleted and embedded in 3% agarose. They were dehydrated through an acetone series to 100% before being placed in EMbed 812 epoxy resin. 80-nm sections (RMC MT-7000 ultramicrotome) were stained with uranyl acetate and lead citrate. The cell profiles were visualized using a Tecnai 12 electron microscope, and photographs of 30–35 nucleated peritoneal MCs were taken from each section.

Morphometric analyses were performed using unbiased stereology techniques. The granule volume fraction (fraction of the total volume of the cell occupied by granules) and surface density (directly proportional to the membrane surface per unit of volume, a measurement of cell and granule shapes) were measured using the point-counting method in a cycloid grid, and the cell profile area (proportional to the volume of the cell) was measured using a point grid.

**Fluorescence Microscopy**—Ears were excised, fixed overnight at 4 °C in 4% paraformaldehyde (pH 7.0), dehydrated, and embedded in paraffin. Avidin binds strongly to the heparin-containing serglycin proteoglycans in the secretory granules of MCs (9). 5- $\mu$ m cross-sections were deparaffinized, rehydrated, incubated with FITC-avidin and Hoechst (Invitrogen) for 1 h at 25 °C, and then mounted with Fluoromount (Diagnostic BioSystems). Images were acquired using a fluorescence microscope with a triple-fluorescence filter (DAPI, GFP, and Texas Red). Taking advantage of the autofluorescence of the cartilage and muscle observed in the red channel, we used the Image-Pro Plus software package to delineate the dermis of the ears as the tissue between the epidermal layers, excluding all muscle and cartilage. All dermal FITC-avidin-positive cells with a Hoechst-positive nucleus were counted. Results are expressed as the number of mature MCs/mm<sup>2</sup> of dermis.

**Derivation, Processing, and Calcium Ionophore and PMA Activation of mBMMCs**—After euthanasia with CO<sub>2</sub>, harvested bone marrow cells from the femora and tibiae of WT and RasGRP4-null mice were cultured for ~6 weeks in medium supplemented with recombinant mouse IL-3 (5 ng/ml; R&D Systems) or IL-3-enriched WEHI-3 cell-conditioned medium as described previously (1). To follow their development, aliquots of  $5 \times 10^4$  non-adherent cells in the culture were analyzed at different time intervals by flow cytometry as described above. The proportion of CD117<sup>+</sup>/Fc $\epsilon$ RI $\alpha$ <sup>+</sup> cells and the mean fluorescence intensity of each fluorophore were determined. mBMMCs ( $1 \times 10^6$ , 6 weeks old) were also lysed in 0.2% Triton X-100, and their histamine content was determined by ELISA (Oxford Biomedical Research).

Although WT mBMMCs constitutively express mouse MC protease (mMCP)-5 and mMCP-6, these cells can be induced to express numerous cytokines and chemokines when exposed to PMA or calcium ionophore A23187. Thus, for the PMA stimulation studies,  $10^6$  WT and RasGRP4-null mBMMCs were placed in separate 24-well culture dishes containing 1.2 ml of 50% WEHI-3-conditioned medium. 3 days later, PMA was added to a final concentration of 125–250 ng/ml. The treated cells were exposed to PMA for 40–120 min. The resulting cells

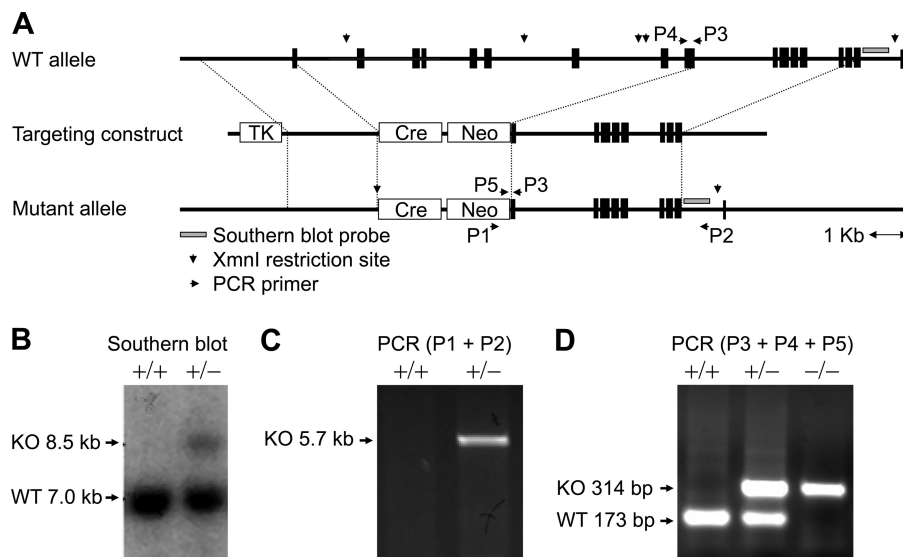
were centrifuged, and an RNeasy mini kit (Qiagen) was used to isolate the RNA in each sample, which was then converted into cDNA using an iScript cDNA synthesis kit (Bio-Rad). Using Qiagen validated primers sets and a Stratagene Mx3000P PCR machine, standard quantitative PCR approaches were carried out to determine the relative levels of the transcripts that encode GAPDH, mMCP-5, mMCP-6, IL-1 $\beta$ , TNF- $\alpha$ , CXCL1, CXCL2, CCL3, and CCL5 in the samples. WT and RasGRP4-null mBMMCs also were exposed to calcium ionophore A23187 (2–8  $\mu$ M; Sigma) for 30 min as described previously (10). Some of the treated cells were used to measure the net percent release of the granule constituent  $\beta$ -hexosaminidase (11). RNA was isolated from the remainder of the untreated and calcium ionophore-treated mBMMCs for quantitative PCR analysis.

***D. farinae*-induced Airway Inflammation**—Pulmonary inflammation was induced in age- and sex-matched WT and RasGRP4-null mice by intranasal instillation of 3  $\mu$ g of *D. farinae* house dust mite extract (GREER Laboratories, Inc., Lenoir, NC) in 20  $\mu$ l of 0.9% NaCl twice a week for 3 weeks as described previously (12, 13). Control mice received only 0.9% NaCl. 24 h after the last treatment, the mice were killed, and bronchoalveolar lavage (BAL) was performed with three aliquots of 0.7 ml of ice-cold PBS containing 10% FCS and 0.5 mM EDTA. The cells in the BAL fluids were counted; cytocentrifuged onto glass slides; stained with Diff-Quick (Fisher), and differentially tallied as macrophages/monocytes, lymphocytes, neutrophils, or eosinophils.

**Dextran Sodium Sulfate (DSS)-induced Colitis**—Male WT and RasGRP4-null mice (8 weeks old) were exposed to a 1.5% solution of DSS (36–50 kDa; MP Biomedicals, Solon, OH) in their drinking water for 7 days as described previously (14, 15). Earlier studies revealed that exposure of WT mice to DSS for 7 days caused an ~10–15% weight loss with minimal mortality but substantial inflammation as measured by histology at day 9. Mice therefore received an additional 2 days of regular drinking water before they were killed at day 9. The colons of the treated and untreated mice were then harvested, and the lengths were measured. The tissue was fixed in formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. The histopathology was evaluated and blindly scored using a 20-point scoring system (16).

**K/BxN Mouse Arthritis Model**—Arthritogenic K/BxN mouse serum containing autoantibodies against glucose-6-phosphate isomerase was administered to recipient WT and RasGRP4-null mice as described previously (17, 18). To induce arthritis, each mouse received 75  $\mu$ l of K/BxN mouse serum intraperitoneally diluted to a final volume of 150  $\mu$ l with LPS-free PBS on experimental days 0 and 2. Before and 30 min after the initial injection, paw swelling was measured using a spring-loaded caliper as described (19) to measure acute edema (“flare”) reflective of MC activation, among other factors. Thereafter, clinical indices were recorded at 48-h intervals and graded on a scale of 0–12. Ankles were harvested as described previously (20) for histochemistry of their arthritic joints. Briefly, the skin around each ankle was gently removed, and the tissue from the distal one-third of the tibia to the mid-paw was collected. After fixation in 4% paraformaldehyde, ankles were decalcified in Kristensen’s solution, dehydrated, and embedded in paraffin. Mid-sagittal ankle sections (5  $\mu$ m) were stained with hematoxylin

## RasGRP4-dependent Arthritis and Colitis



**FIGURE 1. Generation of transgenic RasGRP4-null C57BL/6 mouse line.** *A*, using a homologous recombination approach, the coding sequences of exons 1–8 of the *RasGRP4* gene were deleted starting at the translation initiation site in C57BL/6 mouse ES cells. Herpesvirus thymidine kinase (*TK*) and neomycin resistance (*Neo*) were used for negative and positive selection, respectively. *B*, blot analysis of *XmnI*-digested ES cell genomic DNA was carried out to refine the selection of the transgenic mice. For these analyses, a probe was selected that resided outside the homology arms (gray bar in *A*). *C*, during crossings with WT C57BL/6 mice, the first four generations of the heterozygous mice were screened using a long-range PCR strategy with primers residing inside and outside the targeting construct (P1 + P2). This was done to select for homologous recombination while diluting non-targeted events. *D*, a simpler PCR strategy (P3 + P4 + P5) was used after that for genotyping the generated animals as WT (+/+), knock-out (KO; -/-), or heterozygous (+/-) mice.

and eosin. Histological grading of synovial inflammation and cartilage and bone erosion in the ankle sections was performed as described previously (21).

**Statistical Analysis**—Data are expressed as means  $\pm$  S.E. The normal distribution of the samples was confirmed with the Kolmogorov-Smirnov test before comparing means by Student's two-tailed paired *t* test or series by analysis of variance.

## RESULTS AND DISCUSSION

***RasGRP4* Is Not Essential for Kit Ligand- and IL-3-dependent Development of Mouse MCs**—Using a homologous recombination approach and the targeting construct shown in Fig. 1, an inbred transgenic C57BL/6 mouse line was created that lacked the *RasGRP4* transcript in its MCs. Despite the evolutionary conservation of the *RasGRP4* gene for >75 million years, RasGRP4-null mice were viable and had no obvious developmental defect.

RasGRP1 is essential for the final stages of T cell development in mice (22), and a RasGRP4-defective variant of the HMC-1 cell line derived from a patient with MC leukemia differentiated further when induced to express mouse or human RasGRP4 (3, 8, 23). These data raised the possibility that RasGRP4 might play an important role in the final stages of MC development. Unexpectedly, RasGRP4-null mice contained normal numbers of heparin-positive MCs in their skin (Fig. 2*A*) and peritoneal cavity (Fig. 2, *B–D*, and Table 1). In addition, these *in vivo* differentiated MCs were highly granulated (Fig. 2, *B* and *D*) and expressed normal levels of Kit/CD117 and the high-affinity IgE receptor FcεRI on their plasma membranes (Fig. 2*C*).

The number of granulated MCs in mice is highly dependent on the cytokine Kit ligand and its receptor CD117, and *W/W<sup>v</sup>* mice that have an inactivating mutation in the intracellular

domain of CD117 have very few mature MCs in their tissues (24). The finding that RasGRP4-null mice have normal numbers of mature MCs in their tissues (Fig. 2) indicates that this signaling protein is not essential in the Kit ligand/CD117 signaling pathway needed for MC development in tissues in the C57BL/6 mouse. Nevertheless, the observation that the RasGRP4-defective human MC line HMC-1 became more differentiated when induced to express the signaling protein raises the possibility that mouse MCs might have a guanine nucleotide exchange factor that is not present in human MCs that can compensate for the loss of RasGRP4 expression in the MCs in our transgenic line. If that occurs, the inactivation of RasGRP4 in humans might result in more serious defects in MC development than those found in our RasGRP4-null mice.

Valent *et al.* (7) concluded that MCs and macrophages originate from a common progenitor. Although peripheral blood monocytes express RasGRP4 at the mRNA level (3, 25), no macrophage that contains detectable amounts of RasGRP4 mRNA or protein has been found. Thus, monocytes cease expressing RasGRP4 as they develop into tissue macrophages. In support of this conclusion, the *in vitro* exposure of isolated human monocytes to lectins resulted in rapid decreases in the levels of the *RasGRP4* transcript (3). Although we did not evaluate the functional status of the macrophages in the peritoneal cavities of our RasGRP4-null mice, there was no decrease in their number (Fig. 2). As assessed by Wright-Giemsa staining, 100- $\mu$ l samples of the peritoneal cavity exudates of WT and RasGRP4-null mice contained  $69.4 \pm 3.1\%$  and  $72.2 \pm 2.4\%$  macrophages (mean  $\pm$  S.E., *n* = 5), respectively. Also, there were no differences in the numbers of alveolar macrophages before and after the induction of airway inflammation (Fig. 3).



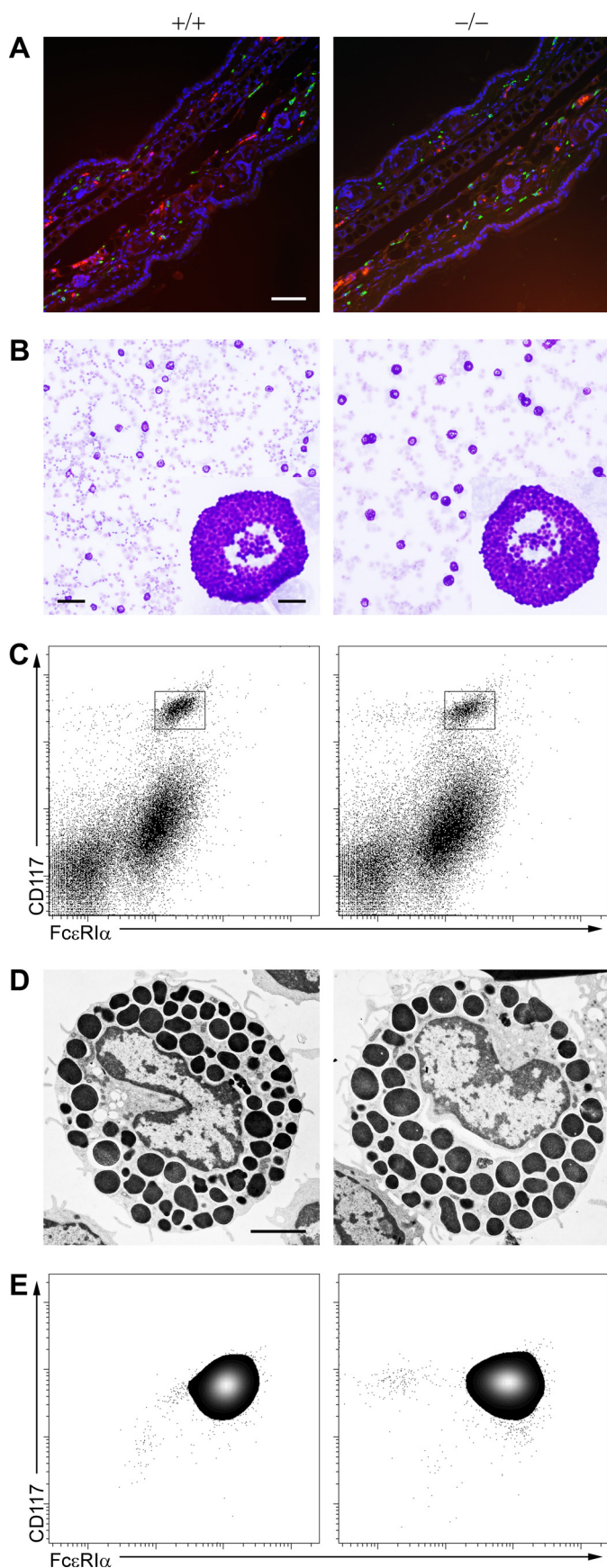


FIGURE 2. Characterization of *in vivo* and *in vitro* differentiated MCs. Shown are representative figures of multiple experiments analyzed in Table 1 from WT (+/+) and RasGRP4-null (-/-) mice. A, ear sections showing MCs

TABLE 1

Quantitation, morphometry, and cellular contents of studied MCs

Values were obtained from five animals of each genotype and are expressed as mean ± S.E.

	RasGRP4+/+	RasGRP4-/-
<b>Skin MCs<sup>a</sup></b>		
No./mm <sup>2</sup> of dermis	247 ± 11	246 ± 11
<b>Peritoneal MC count<sup>b</sup></b>		
%	6.2 ± 0.7	5.7 ± 2.9
10 <sup>3</sup> /ml	138 ± 16	131 ± 67
<b>Peritoneal MCs, flow cytometry<sup>c</sup></b>		
%	3.2 ± 0.4	3.7 ± 0.9
CD117 MFI (10 <sup>3</sup> AU)	33.4 ± 7.5	30.9 ± 6.6
FcεRIα MFI (10 <sup>3</sup> AU)	24.7 ± 9.9	26.1 ± 6.7
<b>Peritoneal MCs, morphometry<sup>d</sup></b>		
V <sub>v</sub>	0.57 ± 0.02	0.60 ± 0.03
S <sub>v</sub> (μm <sup>-1</sup> )	20.57 ± 0.79	21.22 ± 3.90
A (μm <sup>2</sup> )	29.86 ± 0.98	33.04 ± 0.86
<b>mBMMCs in culture (%)<sup>e</sup></b>		
2 weeks	14.4 ± 3.3	16.1 ± 3.9
4 weeks	88.0 ± 5.5	85.5 ± 5.0
6 weeks	98.4 ± 1.9	97.9 ± 1.3
<b>mBMMCs receptors and histamine<sup>f</sup></b>		
CD117 MFI (10 <sup>3</sup> AU)	6.5 ± 3.1	7.3 ± 4.3
FcεRIα MFI (10 <sup>3</sup> AU)	9.8 ± 4.4	10.2 ± 6.1
Histamine (μg/ml)	1.2 ± 0.2	1.4 ± 0.1

<sup>a</sup> FITC-avidin-positive cells with a Hoechst-positive nuclear profile per mm<sup>2</sup> of dermis in random sections of ear (nine samples/animal).

<sup>b</sup> Neubauer chamber counts and Wright-Giemsa staining of cytopsin of peritoneal lavages with 10 ml of PBS (three samples/animal).

<sup>c</sup> Percent CD117<sup>+</sup>/FcεRIα<sup>+</sup> cells in the peritoneal lavages; cell surface expression of these two receptors expressed as mean fluorescence intensity (MFI) in arbitrary units (AU) of specific primary-labeled antibodies bound to the surface of double-positive cells (one sample/animal).

<sup>d</sup> Stereological analysis of randomly acquired electron microscopy images from peritoneal MCs. V<sub>v</sub>, granule volume fraction; S<sub>v</sub>, granule surface density; A, cell profile area (10 cell profiles/sample; five samples/animal).

<sup>e</sup> Percent CD117<sup>+</sup>/FcεRIα<sup>+</sup> cells in mBMMC cultures assessed by flow cytometry (one sample/animal).

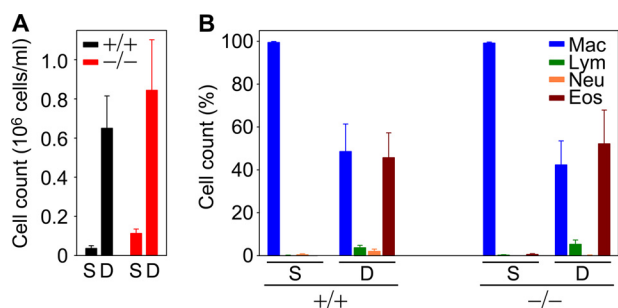
<sup>f</sup> mBMMCs at 6 weeks in culture; cell surface expression of CD117 and FcεRIα measured as MFI; total histamine concentration in lysates of 10<sup>6</sup> mBMMCs measured by ELISA (one sample/animal).

Thus, RasGRP4 also is not essential for the homing and retention of monocytes in tissues and their subsequent differentiation into macrophages.

RasGRP4 was initially cloned from IL-3-dependent mBMMCs (3), raising the possibility that this signaling protein participates in an IL-3-dependent signaling pathway that regulates the development and/or function of MCs. Similar numbers of CD117<sup>+</sup>/FcεRI<sup>+</sup> mBMMCs were generated from WT and RasGRP4-null mice (Fig. 2E and Table 1). Moreover, the *in vitro* differentiated mBMMCs contained comparable amounts of histamine (Table 1). Peritoneal and cutaneous mouse MCs and mBMMCs store the chymase/elastase mMCP-5, the tryptase mMCP-6, and the exopeptidase carboxypeptidase A3 in their granules ionically bound to heparin-containing serglycin proteoglycans. In support of the histochemistry (Fig. 2, A and B) and FACS (Fig. 2C) data, the mBMMCs generated from RasGRP4-null mice contained similar levels of the transcripts that encode mMCP-5

stained with FITC-avidin (green). Hoechst (blue) and the autofluorescence of cartilage and muscle (red) were used to delineate the area of the dermis. Scale bar = 100 μm. B, cytopsin of the cells in peritoneal lavages stained with Wright-Giemsa. Scale bar = 100 μm. Inset, scale bar = 5 μm. C, flow cytometry of cells obtained by peritoneal lavage and labeled with fluorescent antibodies against CD117 and FcεRIα. MCs are the double-positive cells. D, transmission electron microscopy of individual peritoneal MCs used for the unbiased stereology studies. Scale bar = 2 μm. E, flow cytometry of mBMMCs after 6 weeks of culture in IL-3-enriched medium.

## RasGRP4-dependent Arthritis and Colitis



**FIGURE 3. BAL fluid cell counts of mice subjected to *D. farinae* model of airway inflammation.** BAL fluids were obtained from WT (+/+) and RasGRP4-null (-/-) mice ( $n = 7$  per group) 1 day after the last exposure to saline alone (S) or saline containing *D. farinae* house dust mite extract (D). The total number of cells (A) and differential counts (B) in the samples reflect the eosinophilic airway inflammation induced by the house dust mite extract. No significant difference in the number of eosinophils was obtained between the two groups of mice. Mac (blue), macrophages and monocytes; Lym (green), lymphocytes; Neu (orange), neutrophils; Eos (red), eosinophils.

( $n = 10$ ), mMCP-6 ( $n = 10$ ), carboxypeptidase A3 ( $n = 1$ ), and serglycin ( $n = 1$ ) (data not shown).

Cultured mBMMCs that differed in the expression of RasGRP4 were tested for their elaboration of inflammatory mediators once activated. Exposure of WT and RasGRP4-null mBMMCs to 2–8  $\mu\text{M}$  calcium ionophore A23187 for 30 min resulted in the release of comparable amounts of  $\beta$ -hexosaminidase. Moreover, both populations of calcium ionophore-treated MCs markedly increased their levels of the transcripts that encode TNF- $\alpha$  and numerous other cytokines and chemokines (data not shown). Interestingly, when these two cell lines were activated with PMA, a unique inflammatory program that was dependent on the expression of RasGRP4 was observed. For example, the levels of the transcripts that encode the proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  were  $22.1 \pm 9.0$ - and  $5.5 \pm 1.9$ -fold (mean  $\pm$  S.E.,  $n = 5$ ) higher, respectively, in WT mBMMCs exposed to 125 ng/ml PMA for 40–120 min relative to similarly treated RasGRP4-null mBMMCs. In contrast, PMA-treated WT and RasGRP4-null mBMMCs contained similar levels of the transcripts that encode the chemokines CXCL1, CXCL2, CCL3, and CCL5 ( $n = 2$ ).

**RasGRP4 Is Not Essential for *D. farinae*-dependent Eosinophilic Inflammation of Airways**—Extracts of the house dust mite *D. farinae* induce bronchovascular inflammation in mice that is dominated by eosinophils (12, 13). WT and RasGRP4-null mice developed virtually identical increases in the numbers of eosinophils in their BAL fluids 24 h after the final *D. farinae* exposure (Fig. 3). Although there were fewer neutrophils in the BAL fluids of *D. farinae*-treated RasGRP4-null mice relative to similarly treated WT mice, the significance of the data could not be determined due to the fact that neutrophils were not abundant even in the samples from the treated WT mice. Nevertheless, the observation that similar numbers of eosinophils were recruited into the lungs of WT and RasGRP4-null mice in this airway inflammation model suggests that RasGRP4 is not essential in the development and tissue recruitment of eosinophils in mice as anticipated.

**RasGRP4 Contributes to Maximal Inflammation in Acute DSS-induced Colitis Model**—MCs have been implicated in ulcerative colitis and Crohn's disease (26–30), as well as in sev-

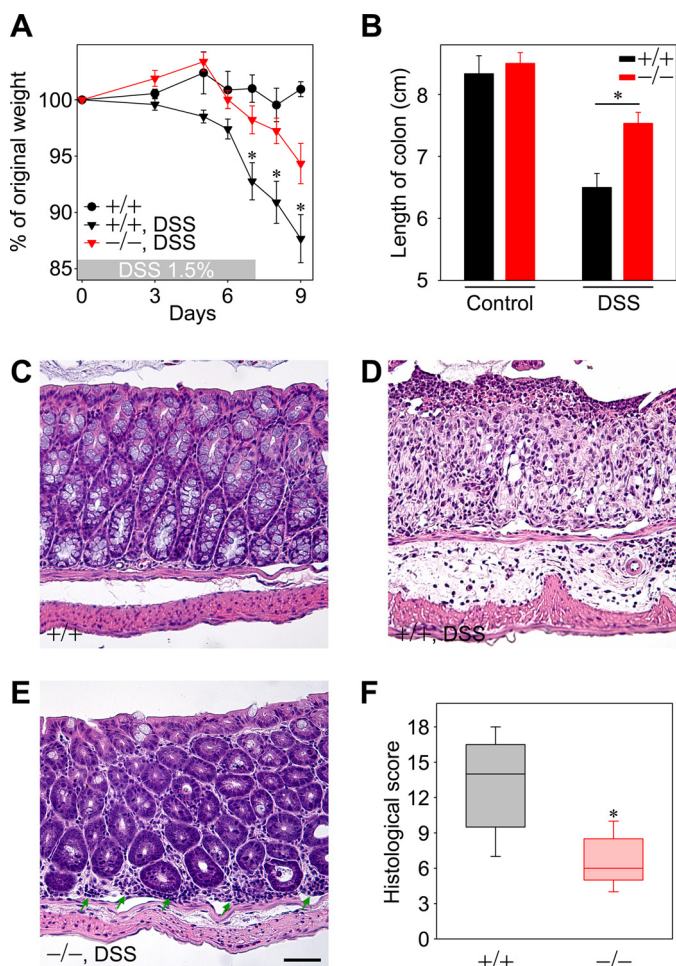
eral rodent models of these inflammatory disorders (15, 31). Oral administration of DSS results in an acute colitis in rodents that replicates many of the features of inflammatory bowel disease in humans (32), and we previously discovered that DSS-induced colitis was markedly reduced in two transgenic mouse strains that lack mMCP-6 (15). Although no defect in the expression of this tryptase was found in the mBMMCs generated from RasGRP4-null mice, the relevant ligand-receptor pathway(s) in the inflamed colon that is needed for the release of this and other proinflammatory mediators has not been identified. The levels of the IL-1 $\beta$  and TNF- $\alpha$  transcripts were increased in the colons of DSS-treated WT mice (15). The finding that the levels of the transcripts that encode IL-1 $\beta$  and TNF- $\alpha$  were reduced in PMA-treated RasGRP4-null mBMMCs raised the possibility that RasGRP4 could regulate the elaboration and release of one or more cytokine and/or granule mediators from MCs in the inflamed colon tissue. Thus, age-matched male WT and RasGRP4-null mice were given a 1.5% solution of DSS in their drinking water for 7 days, followed by 2 days of normal drinking water.

Relative to DSS-treated WT mice, DSS-treated RasGRP4-null mice exhibited reduced colitis as evidenced by less weight loss, longer colons, and lower histopathology scores (Fig. 4). Although inflammatory cells were found in the colons of DSS-treated RasGRP4-null mice, their numbers were considerably fewer than in DSS-treated WT mice. Moreover, the loss of the epithelial lining of the colon was considerably less in these mice.

**K/BxN Arthritis Is Highly Dependent on RasGRP4**—Unlike the *D. farinae*-induced airway inflammation model, neutrophils are the predominant granulocytes found in the colons of DSS-treated mice. The finding that inflammation was reduced in the acute DSS colitis model raised the possibility that RasGRP4 is more important in controlling the accumulation of neutrophils in inflamed tissues than eosinophils. Large numbers of neutrophils are found in the joints of patients with rheumatoid arthritis and in numerous rodent models of this disorder. In the K/BxN arthritis model, mice are given pro-arthritis serum that contains autoantibodies against anti-glucose-6-phosphate isomerase (33, 34). The advantage of this immune complex model over antigen-driven arthritis models (e.g. aggrecan proteoglycan-, type II collagen-, and methylated albumin-induced arthritis) is that adaptive immunity is bypassed, affording the possibility to study the effector phase of IgG-mediated joint inflammation in isolation.

The importance of MCs and their mediators in experimental arthritis is controversial. Although experimental arthritis is not reduced in some MC-deficient mouse lines (35, 36), it is reduced in others (17, 37). In K/BxN arthritis, some of the discrepancy is a consequence of how much arthritogenic antibody is given to each animal and on what day the arthritis is evaluated. mMCP-5, mMCP-6, and mMCP-7 are prominent serine proteases stored in the secretory granules of the MCs in the synovium of BALB/c mice ionically bound to heparin-containing serglycin proteoglycans, and K/BxN mouse serum-induced arthritis and methylated albumin-induced arthritis are both markedly reduced in transgenic C57BL/6 mice that lack these protease-heparin complexes (18, 38). In support of these data,





**FIGURE 4. RasGRP4 deficiency abrogates acute DSS-induced colitis.** A, percent weight loss in the DSS model of acute inflammatory colitis. DSS-treated WT mice (+/+) lost significantly more weight (\*,  $p < 0.05$ ) at days 7, 8, and 9 than similarly treated RasGRP4-null mice (-/-) ( $n = 9$  per group). B, colon length also was reduced to a greater extent (\*,  $p < 0.05$ ) in DSS-treated WT mice compared with similarly treated RasGRP4-null mice ( $n = 9$  per group). Untreated controls ( $n = 3$  per group) are shown. C–F, histopathology of DSS-induced colitis of WT (+/+) and RasGRP4-null (-/-) mice at day 9. Scale bar = 50  $\mu\text{m}$ . The histology of a colon from an untreated WT mouse is depicted in C. It was undistinguishable from the colon of an untreated RasGRP4-null mouse (data not shown). Classic features of DSS-induced colitis in a WT mouse are ulceration, loss of crypts, and inflammatory infiltrate in the lamina propria with lymphocytes, neutrophils, and plasma cells (D). DSS-treated RasGRP4-null mice had intact crypts, minimal damage to the epithelium, and less inflammatory infiltrates (green arrows) (E). The degree of histological inflammation was scored on a 20-point scale (F). DSS-treated WT mice had a more severe score of  $13.2 \pm 1.3$  (mean  $\pm$  S.E.) than DSS-treated RasGRP4-null mice, which had a score of  $6.5 \pm 1.5$  (\*,  $p < 0.005$ ;  $n = 9$  per group). In the box plot, the mid-line indicates the median, the box boundaries indicate the 25th–75th percentile, and error bars indicate the 10th–90th percentile.

the injection of mMCP-6 or its human ortholog tryptase- $\beta$  into varied tissues of naïve WT mice results in the accumulation of large numbers of neutrophils (39–41). MC tryptases also activate synovial procollagenase (42, 43) and other matrix metalloproteinases, which leads, in turn, to the degradation of cartilage proteoglycans (44). Finally, inflammatory arthritis in humans and mice is dependent on the cytokines TNF- $\alpha$  and IL-1 $\beta$ , and evidence has been obtained that MCs contribute to the initiation of inflammation within the joint in the K/BxN arthritis model in part by elaboration of IL-1 $\beta$  (45).

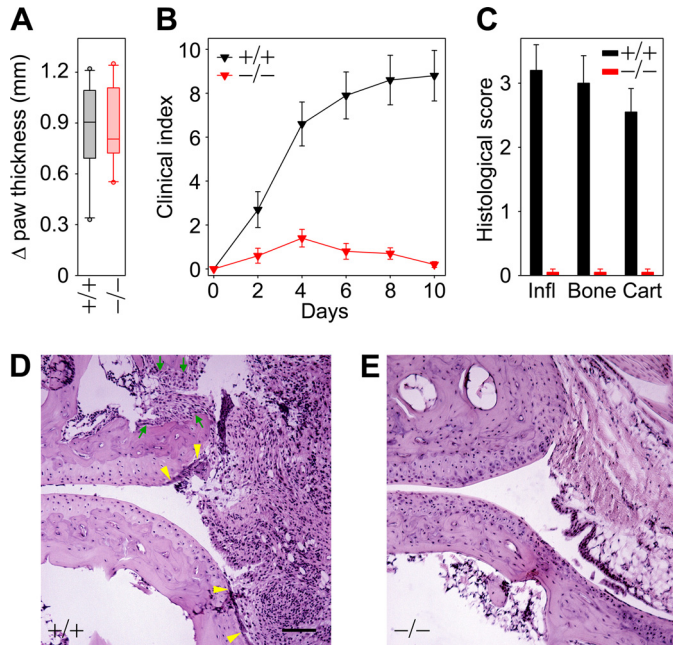
PMA-treated RasGRP4-null mBMMCs contained fewer TNF- $\alpha$  and IL-1 $\beta$  transcripts than similarly treated WT mBMMCs, raising the possibility that RasGRP4 participates in a signaling pathway that results in release of different mediators from MCs and possibly other cell types in the synovium that are needed for inflammation and joint destruction. Thus, RasGRP4-null and WT mice were given diluted K/BxN mouse serum to evaluate the *in vivo* importance of this signaling protein in this acute experimental arthritis model. WT and RasGRP4-null mice had a similar initial serum-induced flare reaction (Fig. 5A), which is an acute transient tissue edema dependent on MCs and neutrophils (19). Despite these data, RasGRP4-null mice remained essentially arthritis-free in the 10-day experiments (Fig. 5, B, C, and E) in contrast to similarly treated WT mice (Fig. 5, B–D). This differential phenotype was not due to accelerated clearance of K/BxN autoantibodies, which were at similar levels in both strains at the time of harvest ( $0.92 \pm 0.26$  versus  $1.07 \pm 0.09$  absorbance units in WT and RasGRP4-null mice, respectively). Thus, RasGRP4 is essential for the effector phase of IgG-mediated murine arthritis.

**Conclusions**—In the mouse, RasGRP4 is not essential for the homing and retention of MC-committed progenitors in tissues, their granule maturation, or their expression of certain surface proteins.<sup>3</sup> Despite these data, RasGRP4-null mBMMCs contained less IL-1 $\beta$  and TNF- $\alpha$  mRNAs when exposed to PMA relative to similarly treated WT mBMMCs. More importantly, experimental colitis and arthritis were both significantly reduced in RasGRP4-null mice. The accumulated data suggest that RasGRP4 participates in an undefined receptor pathway in MCs that is needed for the release of proinflammatory mediators, which ultimately results in the accumulation of neutrophils in inflamed tissues. In support of our arthritis data, Hashimoto *et al.* (25) recently identified a subset of patients with rheumatoid arthritis who had dysregulated expression of RasGRP4 in their peripheral blood mononuclear cells. Although we have not ruled out the possibility that RasGRP4 controls a signaling pathway in another cell type that participates in the animal models used in our study, the accumulated data raise the possibility that the pharmacologic inactivation of this intracellular signaling protein might be of therapeutic benefit in the treatment of neutrophil-predominant inflammatory disorders such as arthritis and inflammatory bowel disease.

<sup>3</sup> The creation of our RasGRP4-null C57BL/6 mouse line and the use of these transgenic mice to demonstrate the importance of the signaling protein in experimental arthritis and colitis were presented at the 75th Annual Scientific Meeting of the American College of Rheumatology (November 5–9, 2011, Chicago), and the relevant abstract was published prior to this scientific meeting (46). While this manuscript was in preparation, Zhu *et al.* (47) independently described the creation of a RasGRP4-null C57BL/6 mouse line that they then mated with a RasGRP1-null mouse line previously created by Dower *et al.* (22). In agreement with the data shown in Fig. 2 and Table 1 of our study, Zhu *et al.* reported that their RasGRP4-null mice contained normal numbers of granulated MCs that expressed Kit and Fc $\epsilon$ RI in the peritoneal cavity and skin. Although the authors showed in their study that RasGRP1<sup>-/-</sup>/RasGRP4<sup>-/-</sup> mBMMCs had a more severe block in Fc $\epsilon$ RI-dependent signaling than RasGRP1<sup>+/+</sup>/RasGRP4<sup>-/-</sup> mice mBMMCs, the latter mice were not subjected to different disease models, as we have done in our study. It therefore remains to be determined whether or not experimental arthritis and colitis are significantly reduced in the RasGRP4-null mice created by Zhu *et al.*



## RasGRP4-dependent Arthritis and Colitis



**FIGURE 5. RasGRP4 deficiency abrogates K/BxN arthritis.** *A*, change in combined paw swelling 30 min after intraperitoneal injection of the first dose of K/BxN mouse serum demonstrating equivalent flare in WT (+/+) and RasGRP4-null (-/-) mice. In the box plot, the *mid-line* indicates the median, the *box boundaries* indicate the 25th–75th percentile, *error bars* indicate the 10th–90th percentile, and *circles* indicate the range. *B*, arthritis severity after intraperitoneal injection of the serum on days 0 and 2 showing significant lack of clinical deterioration in RasGRP4-null mice ( $n = 10$  mice/group;  $p < 0.0001$ , analysis of variance). *C*, histological scoring of ankle sections at day 10 showing minimal inflammation (*Infl*) and preservation of integrity of articular bone and cartilage (*Cart*) in RasGRP4-null mice ( $n = 20$ /group;  $p < 0.0001$  for all categories). *D* and *E*, histology of midsagittal sections of the anterior tibiotalar joint obtained at day 10 from WT (+/+; *D*) and RasGRP4-null (-/-; *E*) mice. Scale bar = 100  $\mu\text{m}$ . Representative sections demonstrate inflammatory changes in WT but not RasGRP4-null mice, including a dense cellular infiltrate into the synovium, synovial adhesion to cartilage margins with associated surface injury (yellow arrowheads), and erosion through cortical bone (green arrows).

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