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Netrin-1 Signaling Dampens Inflammatory Peritonitis

Valbona Mirakaj,*† Dimitra Gatidou,* Claudia Pötzscher Klemens König,† and Peter Rosenberger*†

Previous studies implicated the anti-inflammatory potential of the adenosine 2B receptor (A2BAR). A2BAR activation is achieved through adenosine, but this is limited by its very short t1/2. To further define alternative adenosine signaling, we examined the role of netrin-1 during acute inflammatory peritonitis. In this article, we report that animals with endogenous repression of netrin-1 (Ntn1–/–) demonstrated increased cell count, increased peritoneal cytokine concentration, and pronounced histological changes compared with controls in a model of zymosan A peritonitis. Exogenous netrin-1 significantly decreased i.p. inflammatory changes. This effect was not present in animals with deletion of A2BAR (A2BAR–/–), A2BAR–/– animals demonstrated no change in cell count, i.p. cytokine concentration, or histology in response to netrin-1 injection. These data strengthen the role of netrin-1 as an immunomodulatory protein exerting its function in dependence of the A2BAR and further define alternative adenosine receptor signaling. The Journal of Immunology, 2011, 186: 000–000.

The online version of this article contains supplemental material.

Materials and Methods

Transcriptional and protein analysis

Murine transcriptional analysis of Ntn1 mRNA was performed using sense primer 5′-GAG CGG GGG AGT CTG TCT-3′ and antisense primer 5′-TGG TTT GAT GGC TTT AGG TCT-3′. Murine β-actin expression was evaluated using sense primer 5′-CTC TCC TCT CTC AGG CCA TCC TG-3′ and antisense primer 5′-TCA CGC ACG ATT TCC CTC G-3′. Semiquantitative analysis of human Ntn1 mRNA expression was performed using primer sets containing sense primer 5′-GAG TGC GGC GTC CAC-3′ and the antisense primer 5′-AGG CAG ACA CCT CCG TTC TT-3′. Samples were controlled for β-actin using the following primers: sense 5′-GCT GGC TTT TAG GAT GCC AAG-3′, antisense 5′-ACT...
GGA ACG GTG AAG GTG ACA G-3’. All PCR analyses were performed using real-time PCR (iCycler; Bio-Rad, Hercules, CA), as described previously (12).

For Western blot analysis, animal samples were homogenized, normalized for protein levels, and applied in nonreducing conditions to SDS containing polyacrylamide gels. Abs used for Western blotting included rabbit polyclonal anti–netrin-1 (sc 20786; Santa Cruz Biotechnology, Santa Cruz, CA) for murine Ntn1 analysis. Actin was stained using rabbit anti–Actin (Cell Signaling Technology, Beverly, MA). Blots were washed, and species-matched peroxidase-conjugated secondary Ab (Santa Cruz Biotechnology) was added. Labeled bands from washed blots were detected by ECL (Amersham Biosciences, Piscataway, NJ).

**Immunofluorescent staining**

For murine immunofluorescence, animals were sacrificed; colon and peritoneum were embedded in paraffin; and tissue sections were placed on slides, air-dried, and fixed in methanol followed by 4% acetone. Air-dried tissue sections were washed three times in PBS and blocked with 5% BSA (Sigma-Aldrich, St. Louis, MO). Rabbit polyclonal anti–netrin-1 (sc 20786; Santa Cruz Biotechnology), at a dilution of 1:1,000, was used as the primary Ab. Texas Red 545-conjugated goat anti-rabbit (Invitrogen, Eugene, OR) was used as the secondary Ab. DAPI (Invitrogen) was used for nuclear counterstaining.

**Histopathological evaluation**

Following peritonitis, mice were killed, and tissues were fixed in 10% formaldehyde solution. Tissues were embedded in paraffin and stained with H&E. Fluorescence microscope LSM 510 Meta (Carl Zeiss, Jena, Germany) was used for imaging.

**Peritonitis model**

All animal experiments were in accordance with the regulations of the Regierungspräsidium Tübingen and the local ethical committee. Mice were injected i.p. with 1 ml ZyA (1 mg/ml; Sigma-Aldrich), and subsequently with vehicle (saline + 0.2% BSA) or 1 μg recombinant murine netrin-1 (R&D Systems, Minneapolis, MN) in 150 μl total volume. Recruited leukocytes were obtained at the described time points by peritoneal lavage with calcium- and magnesium-free ice-cold PBS solution containing 10 U/ml unfractionated heparin. Collected cells were washed, resuspended in 2 ml HBSS, and counted, and cytospin samples were prepared. All reagents used were endotoxin-free.

**Wild-type, Ntn1+/−, and A2BAR−/− animals**

Approval of the Institutional Review Board and the Regierungspräsidium Tübingen was obtained. Wild-type (WT), Ntn1+/−, and A2BAR−/− mice and littermate control mice were bred and genotyped, as described previously (13).

**Measurement of serum cytokines**

TNF, IL-1β, IL-6, and IL-8 were measured in the peritoneal lavage of WT, Ntn1+/−, and A2BAR−/− animals by standard ELISA (R&D Systems).

**Measurement of peritoneal cAMP**

After peritoneal lavage was obtained, cAMP concentrations were determined with a cAMP EIA Kit (Cayman Chemical, Ann Arbor, MI).

**Transcriptional analysis of TNF-α and IL-6**

Human CaCo-2 cells were incubated with ZyA (100 μg/ml) for 12 h to determine transcriptional response of TNF-α and IL-6. Netrin-1 (1 μg/ml) was added, or cells were preincubated with the A2BAR antagonist 1-propyl-8-(2-sulfophenyl)xanthine (10 μM; Sigma-Aldrich) for 30 min prior to netrin-1 addition. Transcriptional analysis of TNF-α was performed using primer sense 5′-TGC TTG TTC TTC AGC TTC TT-3′ and antisense 5′-GGT GGC ACA GCT TGG TTG CA-3′; IL-6 primer used was sense 5′-AGC GCC TTC GGT CGA GTT GC-3′ and antisense 5′-GGT GCT TGC GTC TGT GGG CG-3′.

**Transcriptional analysis of netrin-1R expression**

Murine tissues were obtained from the liver and the intestine to determine the expression levels of A2BAR using sense: 5′-GGA AGG ACT TCG TCT CTC CA-3′ antisense: 5′-GGG CAG CAA CTC AGA AAA CT-3′, neogenin sense: 5′-GCATAACCTGGACACAAAT-3′ antisense: 5′-GCT GCT CTC ACA GTC AAT GG-3′, and UNC5b sense: 5′-GGA CCT CCT CAG TGC TAC A-3′ antisense: 5′-GCT AAG TCC TGC CAC ATC CT-3′ primer in mice.

**Data analysis**

All data are presented as mean ± SEM from 9–11 animals per condition. GraphPad 5.0 software (GraphPad, San Diego, CA) was used for statistical analysis. We performed statistical analysis using one-way ANOVA to determine group differences using post hoc analysis, followed by the unpaired Student t test (two sided, α < 0.05). A p value <0.05 was considered statistically significant.

**Results**

Netrin-1 is repressed during an acute inflammatory response

We initially addressed the question of whether netrin-1 is expressed outside the CNS in intestinal organs to validate the model subsequently used; we found a robust expression of netrin-1 within several murine organs (Fig. 1A, 1B). We then aimed to identify whether netrin-1 expression is affected during an acute inflammatory process CTC ACA GTC AAT GG-3′, and UNC5b sense: 5′-GGA CCT CCT CAG TGC TAC A-3′ antisense: 5′-GCT AAG TCC TGC CAC ATC CT-3′ primer in mice.

**FIGURE 1.** Netrin-1 is expressed outside the CNS. A, Ntn1 mRNA expression in the brain (Br) and outside the CNS in lung (Lu), liver (Li), spleen (Sp), and intestine (In). B, Pooled Western blot analysis of four independent animals demonstrating Ntn1 expression in brain (Br), lung (Lu), liver (Li), spleen (Sp), and intestine (In). C, Immunofluorescent localization of Ntn1 expression (red) in colon of WT animals 4 h following i.p. injection of NaCl 0.9% or ZyA (original magnification ×400). D, Ntn1 protein analysis in WT animals 4 h following i.p. injection of NaCl 0.9% or ZyA. E, Ntn1 mRNA expression in CaCo-2 cells exposed to increasing concentrations of TNF-α for 24 h. F, Ntn1 mRNA expression in CaCo-2 cells exposed to increasing concentrations of IL-6 for 24 h. Data are mean ± SEM (n = 4 per condition). **p < 0.01; ***p < 0.001.
in the peritoneal cavity in vivo and found a significant reduction in netrin-1 in response to sterile peritonitis induced through ZyA (Fig. 1C, 1D). To corroborate this finding, we tested whether netrin-1 expression was changed following inflammatory stimulation in vitro and exposed the intestinal CaCo-2 cell line to TNF-α and IL-6 stimulation. This corroborated that netrin-1 expression is significantly reduced in intestinal epithelial cells in response to inflammatory cytokines (Fig. 1E, 1F).

Endogenous netrin-1 controls acute inflammatory peritonitis

We then proceeded to investigate the functional role of endogenous netrin-1 repression and its role during acute inflammatory peritonitis. For this purpose, we exposed previously characterized Net1+/− mice (Net1−/− are not viable) and initially determined the expressional levels of netrin-1 receptors in these animals but did not find a significant difference between Net1+/+ and Net1+/− animals (Supplemental Fig. 1). Following i.p. ZyA injection, we found that Net1+/− mice exhibited significantly increased cell numbers in the peritoneal lavage compared with Net1+/+ animals (310⁶, 4.2 ± 0.29 versus 2.9 ± 0.39, respectively; p < 0.01) (Fig. 2A). This result was reflected when evaluating the myeloperoxidase (MPO) activity within the peritoneal fluid and the protein content within the peritoneal exudate (Fig. 2B, 2C). We also performed histological evaluation of the peritoneal cavity, the mesenterial fat, and the cellular exudate. We found that Net1+/− mice exhibited significantly pronounced inflammatory changes and tissue destruction of the peritoneal tissue and increased infiltration of inflammatory cells into the mesenterial fat tissue compared with Net1+/+ animals (Fig. 2D). Cytospin samples verified the increased cell count in the peritoneal lavage of Net1+/+ mice following ZyA-induced peritonitis compared with Net1+/− animals. When evaluating inflammatory cytokines within the peritoneal lavage, Net1+/+ mice exhibited significantly increased levels (pg/ml) of TNF-α, IL-1β, IL-6, and IL-8 compared with Net1+/− animals (67 ± 10 versus 38 ± 6, p < 0.01; 1322 ± 445 versus 762 ± 78, p < 0.05; 2479 ± 696 versus 1369 ± 270, p < 0.05; and 4993 ± 836 versus 3268 ± 648, p < 0.05) (Fig. 3). Intraperitoneal levels of cAMP were also significantly different between Net1+/+ and Net1+/− animals (Supplemental Fig. 2).

Exogenous netrin-1 dampens inflammatory peritonitis

We next investigated the functional impact of exogenous netrin-1. We used WT animals and exposed them to ZyA peritonitis, with subsequent injection of vehicle or netrin-1. In the animals injected with netrin-1, we found a significantly reduced cell count (∗10⁶) in the peritoneal lavage compared with vehicle-injected animals (NaCl: 0.59 ± 0.07; 2.24 ± 0.15 versus 3.83 ± 0.25, respectively, p < 0.01) (Fig. 4A). This result was corroborated by the measurement of MPO activity within the peritoneal fluid and the protein content in the peritoneal lavage (Fig. 4B, 4C). In the histological sections of these animals, the peritoneal tissue and the mesenterial fat of animals injected with netrin-1 demonstrated reduced signs of inflammation compared with vehicle controls (Fig. 4D). Cytospin samples of the peritoneal lavage confirmed this finding demonstrating reduced cell count in the netrin-1–treated animals compared with vehicle controls. We then

![FIGURE 2](http://www.jimmunol.org/)

Endogenous netrin-1 dampens inflammatory peritonitis. Net1+/− and Net1+/+ animals were injected i.p. with NaCl 0.9% or 1 mg of ZyA; samples were taken after 4 h. Cell count (A), MPO activity (B), protein content (C), and representative histological evaluation of the peritoneum, the mesenterial fat, and cytospin samples (D) in the peritoneal lavage. Sections were stained with H&E (original magnification ×400; insets ×1000). Data are mean ± SEM (n = 11 per group). *p < 0.05; **p < 0.01; ***p < 0.001.
evaluated inflammatory cytokines within the peritoneal lavage and found that exogenous netrin-1 significantly reduced the concentration (pg/ml) of TNF-α (Fig. 5A), IL-1β (Fig. 5B), IL-6 (Fig. 5C), and IL-8 (Fig. 5D) compared with vehicle treatment (NaCl: 3 ± 2, 15.5 ± 4 versus 32 ± 5, *p* < 0.05; NaCl: 37 ± 23, 435 ± 56 versus 1897 ± 650, *p* < 0.05; NaCl: 6 ± 2, 860 ± 318 versus 2965 ± 617, *p* < 0.05; and NaCl: 16 ± 5, 1467 ± 476 versus 3823 ± 930, *p* < 0.05, respectively). Intraperitoneal levels of cAMP were significantly reduced in the ZyA+Netrin-1 group compared with the ZyA+Vehicle group (Supplemental Fig. 3).

**Exogenous netrin-1 does not reduce inflammatory peritonitis in A2BAR−/− animals**

First, we determined the expressional levels of potential netrin-1Rs in A2BAR−/− animals to exclude the influence of A2BAR deletion. We found a significant difference only for A2BAR expression in these animals, not for other potential netrin-1Rs (Supplemental Fig. 4). To evaluate whether the effect of netrin-1 during an exudative inflammatory process is dependent on A2BAR, we induced ZyA peritonitis in A2BAR−/− animals and injected them with vehicle or netrin-1. There was no change in the cell count (× 10⁶) within the peritoneal lavage of animals injected with netrin-1 compared with vehicle-treated animals (NaCl: 0.25 ± 0.02; 3.83 ± 0.48 versus 3.68 ± 0.39, respectively) (Fig. 6). There also was no reduction in MPO activity within the peritoneal cavity following injection of netrin-1 in A2BAR−/− animals (Fig. 6B, 6C). Histological sections did not exhibit a reduction in inflammation within the peritoneal cavity or the...
mesenterial fat following netrin-1 injection in A2BAR^{2/2} animals (Fig. 6D). Cytospin samples of the peritoneal lavage confirmed this by demonstrating no change in cell count in the netrin-1–injected animals compared with vehicle controls. Finally, when evaluating the concentration of inflammatory cytokines within the peritoneal lavage, we did not find a change in cytokine concentration following exogenous netrin-1 administration in the A2BAR^{2/2} animals. The concentration (pg/ml) of TNF-α (Fig. 7A), IL-1β (Fig. 7B), IL-6 (Fig. 7C), or IL-8 (Fig. 7D) did change following netrin-1 injection compared with vehicle treatment (NaCl: 2.5 ± 0.5, 38 ± 5 versus 38 ± 11; NaCl: 12 ± 4, 545 ± 115 versus 458 ± 90; NaCl: 4 ± 1, 4712 ± 1126 versus 4696 ± 1253; and NaCl: 16 ± 3, 6475 ± 1361 versus 5886 ± 1409, respectively. Intraperitoneal levels of cAMP reflected these results (Supplemental Fig. 5).

To gain additional data about the anti-inflammatory role of netrin-1, we stimulated CaCo-2 cells with ZyA, which resulted in a significant induction of TNF-α and IL-6 transcripts within these cells. When adding netrin-1 to this, we found a significant reduction in the transcriptional response to ZyA. Following pre-incubation of CaCo-2 cells with the A2BAR antagonist 1-propyl-8-(p-sulfophenyl)xanthine, this effect was significantly attenuated but not completely abolished (Supplemental Fig. 6).

**Discussion**

Signaling through the A2BAR possesses anti-inflammatory potential and dampens inflammatory tissue damage. Endogenous activation of A2BAR occurs through adenosine, but recent reports

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**FIGURE 5.** Exogenous netrin-1 dampens release of inflammatory cytokines in vivo. WT animals were injected i.p. with NaCl 0.9% or 1 mg of ZyA, followed by i.v. injection of vehicle or recombinant netrin-1 (1 μg); samples were taken after 4 h. TNF-α (A), IL-1β (B), IL-6 (C), and IL-8 (D) concentrations in peritoneal lavage. Data are mean ± SEM (n = 9 per group). *p < 0.05; **p < 0.01; ***p < 0.001.

**FIGURE 6.** Exogenous netrin-1 does not reduce inflammatory peritonitis in A2BAR^{2/2} animals. A2BAR^{2/2} animals were injected i.p. with NaCl 0.9% or 1 mg of ZyA, followed by i.v. injection with vehicle or recombinant netrin-1 (1 μg); samples were taken after 4 h. Cell count (A), MPO activity (B), protein content (C), and representative histological analysis of the peritoneum, the mesenterial fat tissue, and cytospin samples (D) in the peritoneal lavage. Sections were stained with H&E (original magnification ×400; insets ×1000). Data are mean ± SEM (n = 9 per group). **p < 0.01; ***p < 0.001.
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suggested a role for the neuronal repellent netrin-1 in A2BAR activation. Therefore, we assessed the role of exogenous and endogenous netrin-1 in a model of inflammatory peritonitis to further define its anti-inflammatory role, as well as its potential to exert its function in dependence of the A2BAR. We report in this article that netrin-1 possesses significant anti-inflammatory potential during exudative inflammation, and this effect is dependent on the A2BAR.

The role of netrin-1 was initially described in the context of axonal growth and migration. Floor plate cells at the ventral midline of the mammalian embryonic neural tube secrete netrin-1 and generate a circumferential gradient of netrin-1 protein in the neuroepithelium (25). This protein gradient possesses bifunctional potential, in that it attracts some axons to, as well as repels other axons from, the ventral midline. Subsequent studies demonstrated the expression of netrin-1 outside the nervous system within the vasculature, a reduction in netrin-1 expression during an acute inflammatory process, and that netrin-1 is a potent inhibitor of myeloid cell migration toward chemotactic stimuli in vitro (12, 26). This anti-inflammatory potential of netrin-1 was corroborated by Wang et al. (27) during renal ischemia–reperfusion injury. In this study, netrin-1 stimulation resulted in an increase of an acute inflammatory response in dependence of the A2BAR. Furthermore, netrin-1 significantly reduced the release of inflammatory cytokines; this was not observed in animals with gene-targeted deletion of A2BAR. Given the findings by McKenna et al. (36), a role for A2BAR in the expression of other known netrin-1Rs targeted deletion of A2BAR. Given the findings by McKenna et al. (36), a role for A2BAR in the expression of other known netrin-1Rs on immunocompetent cells might be possible and explain our findings; however, further studies are needed. The results that we presented in this article corroborate an important role for A2BAR for the anti-inflammatory potential of netrin-1.

In summary, our results strengthen the role of netrin-1 as an endogenous modulator of an inflammatory response during an acute exudative inflammation. Exogenous netrin-1 exerts significant anti-inflammatory that is dependent on the A2BAR. As such, these findings increase our knowledge about a possible role for adenosine receptor activation during netrin-1 anti-inflammation.

Disclosures

The authors have no financial conflicts of interest.

References


FIGURE 7. Exogenous netrin-1 does not reduce cytokine release in A2BAR−/− animals. A2BAR−/− animals were injected i.p. with NaCl 0.9% or 1 mg of ZyA, followed by i.v. injection with vehicle or recombinant netrin-1 (1 µg); samples were taken after 4 h. TNF-α (A), IL-1β (B), IL-6 (C), and IL-8 (D) concentration in peritoneal lavage. Data are mean ± SEM (n = 9 per group). **p < 0.01; ***p < 0.001.


