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Netrin-1 Signaling Dampens Inflammatory Peritonitis

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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 activation of immune cells and the subsequent inflammatory response are critical steps during immune surveillance and host defense facilitating the elimination of foreign or infectious agents (1–4). Leukocyte migration and the control of an inflammatory response are tightly controlled multistep processes and have been well characterized in recent years (4, 5). Control mechanisms exist to prevent an exaggerated inflammatory response that might result in exaggerated tissue destruction and subsequent organ dysfunction. Only recently, neuronal-guidance proteins were identified to be part of these control mechanisms and, as such, to attenuate leukocyte infiltration and the associated cytokine release (6–10). The neuronal-guidance protein netrin-1 is involved in the control of local inflammation during tissue hypoxia and the early stages of acute lung injury, and its anti-inflammatory function is dependent on the interaction of netrin-1 with the adenosine 2B receptor (A2BAR) (11–13). This opens the possibility of endogenous adenosine receptor activation independent of the short-lived nucleoside adenosine.

Several reports demonstrated a protective role for adenosine and reported the tissue-protective potential of A2BAR activation. As such, signaling through A2BAR dampens pulmonary inflammation and controls fluid exchange during acute lung injury (14, 15). During ischemia–reperfusion injury, signaling through A2BAR reduces the associated reperfusion injury and possesses protective potential (16, 17). The coordinated induction of A2BAR during cellular hypoxia and an acute inflammatory process has to be seen as an adaptation of the affected tissues to increase vascular barrier response and to induce tissue-protective signaling through the A2BAR (18–21). However, a major limitation of adenosine signaling is that once adenosine is generated into the extracellular milieu, it is also rapidly cleared through passive or active uptake. This occurs through nucleoside transporters, termed equilibrative nucleoside transporters and concentrative nucleoside transporters, respectively. As a result, adenosine action is marked by its very short t1/2 (22, 23). Systemic administration of adenosine as an anti-inflammatory drug is not possible because this results in a variety of undesirable side effects, such as bradycardia or vasodilation (24). Therefore, mechanisms that are able to signal through the A2BAR are highly desirable and need further definition.

Given the role of netrin-1 during hypoxic inflammation, we aimed to define its role during acute exudative inflammation and corroborate a possible role for the A2BAR. In this article, we report that netrin-1 is expressed in intestinal organs, and this expression is reduced during acute peritoneal inflammation. Animals with endogenous repression of netrin-1 (Ntn1−/−) demonstrated an increased inflammatory response following zymosan A (ZyA)-induced peritonitis compared with littermate controls. The injection of exogenous netrin-1 significantly dampened the extent of inflammatory peritonitis; this was dependent on the A2BAR. Therefore, these data corroborate the role of netrin-1 as an endogenous anti-inflammatory protein with significant influence on inflammatory control that is dependent on the A2BAR.

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′-TTG TTT GAT TGC AGG TCT TT-3′. Murine β-actin expression was evaluated using sense primer 5′-CTC TCC CTC ACG CCA TCC TG-3′ and antisense primer 5′-TCA CGG ACG AAT TCC TCC TCA G-3′. Semi-quantitative analysis of human Ntn1 mRNA expression was performed using primer sets containing sense primer 5′-GAG TGC TGC GCC TGT AAC-3′ and the antisense primer 5′-AGG CAG ACA CCT CGG CTC TT-3′. Samples were controlled for β-actin using the following primers: sense 5′-GTT GGC TTT TAG GAT GGC AAG-3′, antisense 5′-ACT
GGA ACG GTG AAG GTG ACA A-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 1:1000, 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 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-(3-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 TCT TTC AGC TTC TT-3′ and antisense 5′-TGG GGT ACA GGC TTG TCA CT-3′; IL-6 primer used was sense 5′-AGC GGT TCT GGT GCA GTT GC-3′ and antisense 5′-GTC GGT GCT GCT GTG 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′ and antisense: 5′-GGG CAG CAA CTC AGA AAA CT-3′; neo-genin sense: 5′-GCA AAT CTCCCGGA CACC-3′ and antisense: 5′-GCT CTC ACA GTC AAT GG-3′, and UNC5b sense: 5′-GGA CCT CAG TGC TAC A-3′ and antisense: 5′-GCT AAG TCC TGC CAC ATC CT-3′ 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.
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 Ntn1+/− mice (Ntn1−/− are not viable) and initially determined the expressional levels of netrin-1 receptors in these animals but did not find a significant difference between Ntn1+/+ and Ntn1+/- animals (Supplemental Fig. 1). Following i.p. ZyA injection, we found that Ntn1+/- mice exhibited significantly increased cell numbers in the peritoneal lavage compared with Ntn1+/+ animals (3.10^6, 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 Ntn1+/- 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 Ntn1+/+ animals (Fig. 2D). Cytospin samples verified the increased cell count in the peritoneal lavage of Ntn1+/- mice following ZyA-induced peritonitis compared with Ntn1+/+ animals. When evaluating inflammatory cytokines within the peritoneal lavage, Ntn1+/- mice exhibited significantly increased levels (pg/ml) of TNF-α, IL-1β, IL-6, and IL-8 compared with Ntn1+/+ 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). Intrapерitoneal levels of cAMP were also significantly different between Ntn1+/+ and Ntn1+/- 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 (3.10^6) 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
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). Intrapерitoneal 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. 6A). 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

**FIGURE 3.** Endogenous netrin-1 dampens inflammatory cytokine release in vivo. Ntn1+/− and Ntn1+/+ animals were injected i.p. with NaCl 0.9% or 1 mg of ZyA; samples were taken after 4 h. TNF-α (A), IL-1β (B), IL-6 (C), and IL-8 (D) concentration in peritoneal lavage of Ntn1+/+ and Ntn1+/− animals. Data are mean ± SEM (n = 11 per group). *p > 0.05; **p < 0.01; ***p < 0.001.

**FIGURE 4.** Exogenous netrin-1 dampens inflammatory peritonitis in vivo. WT 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 < 0.05; **p < 0.01; ***p < 0.001.
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-\(\alpha\) (Fig. 7A), IL-1\(\beta\) (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-\(\alpha\) 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 \(\mu\)g); samples were taken after 4 h. TNF-\(\alpha\) (A), IL-1\(\beta\) (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 \(^{-/-}\) 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 \(\mu\)g); 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 \(\times 400\); insets \(\times 1000\)). Data are mean ± SEM (\(n = 9\) per group). ** \(p < 0.01\); *** \(p < 0.001\).
macrophage activation, thereby preventing tissue injury after episodes of hypoxia and ischemia (32, 33). Thus, A2BAR might be an important pharmacological target in treating conditions associated with barrier dysfunction and acute inflammation. Although investigations of pharmacological approaches for A2BR activation are underway, a possible endogenous activation of A2BAR through netrin-1 has not been well investigated. The present study demonstrated that netrin-1 does not exert anti-inflammatory function in A2BAR−/− animals and, therefore, showed that its function is dependent on A2BAR.

For central nervous function of netrin-1, the deleted in colorectal cancer receptor and the UNC5 homologs UNC5A, -B, -C, and -D were initially reported (34). Corset et al. (11) subsequently demonstrated that netrin-1 also signals through A2BAR during axonal outgrowth. In this study, netrin-1 stimulation resulted in an intracellular cAMP increase that was dependent on A2BAR. However, other reports implicated that the repulsive function of netrin-1 during axonal growth is independent of A2BAR function; rather, it is mediated through the deleted in colon cancer receptor (35). In a study by McKenna et al. (36), the authors demonstrated that activation or overexpression of A2BAR reduced the level of the repellent UNC5A receptor on the surface of neuronal cells. This resulted in a conversion of repulsion to attraction of these cells. Ly et al. (26) demonstrated that the anti-inflammatory function of netrin-1 was dependent on a member of the UNC family, the UNC5b receptor in vitro through chemotactic migration studies, but they did not translate this finding into in vivo evidence. In recent studies, we demonstrated that netrin-1 dampens hypoxic inflammation through A2BAR and corroborated this during acute lung injury (12, 13). In the present study, we investigated the role of netrin-1 during acute exudative inflammation and assessed its role during inflammatory peritonitis. We found that netrin-1 significantly reduced the number of inflammatory cells and dampened the histological changes 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 and, therefore, 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-inflammation 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.

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