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**Graphical abstract**

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Adenosine A₃ agonists reverse neuropathic pain via T cell–mediated production of IL-10

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The A₃ adenosine receptor (A₃AR) has emerged as a therapeutic target with A₃AR agonists to tackle the global challenge of neuropathic pain, and investigation into its mode of action is essential for ongoing clinical development. Immune cell A₃ARs, and their activation during pathology, modulate cytokine release. Thus, the use of immune cells as a cellular substrate for the pharmacological action of A₃AR agonists is enticing, but unknown. The present study discovered that Rag-KO mice lacking T and B cells, as compared with WT mice, are insensitive to the anti-allodynic effects of A₃AR agonists. Similar findings were observed in interleukin-10– and interleukin-10 receptor knockout mice. Adoptive transfer of CD4⁺ T cells from WT mice infiltrated the dorsal root ganglion (DRG) and restored A₃AR agonist-mediated anti-allodynia in Rag-KO mice. CD4⁺ T cells from Adora3-KO or Il10-KO mice did not. Transfer of CD4⁺ T cells from WT mice, but not Il10-KO mice, into Il10-KO mice or Adora3-KO mice fully reinstated the anti-allodynic effects of A₃AR activation. Notably, A₃AR agonism reduced DRG neuron excitability when cocultured with CD4⁺ T cells in an IL-10–dependent manner. A₃AR action on CD4⁺ T cells infiltrated in the DRG decreased phosphorylation of GluN2B-containing N-methyl-D-aspartate receptors at Tyr1472, a modification associated with regulating neuronal hypersensitivity. Our findings establish that activation of A₃AR on CD4⁺ T cells to release IL-10 is required and sufficient evidence for the use of A₃AR agonists as therapeutics.

Introduction

Chronic neuropathic pain constitutes a large unmet medical need affecting 15–30 million people in the United States (1), and the annual economic burden cannot be underscored (2). Neuropathic pain arises when peripheral nerves are injured by trauma, disease, or toxins. Neuropathic pains are chronic, severe, debilitating, and exceedingly difficult to treat with currently available analgesics (3). Novel nonnarcotic analgesics are needed. Recently, the Gi-coupled A₃ adenosine receptor (A₃AR) was identified as a beneficial target in traumatic nerve injury–induced neuropathic pain (4–6). Continued investigation into its mode of action is essential, as these are in clinical development. Immune cell A₃AR levels (7), but whether these receptors play a role in the beneficial agonist effects in neuropathic pain is unknown. Interestingly, A₃AR activation on circulating immune cells harvested from animal models of autoimmune disorders blocks the formation of neuroexcitatory/inflammatory cytokines such as TNF and interleukin IL-10 and enhances interleukin-10 (IL-10) release (8). Similar findings were obtained with immune cells harvested from patients with autoimmune disorders validating the target in humans (9, 10). IL-10 is a potent anti-inflammatory and neuroprotective cytokine with documented positive effects in mitigating neuropathic pain (11, 12). These data, in parallel fields of studies, point to a potential link between immune cells and IL-10 in A₃AR agonist action. Using behavioral, genetic, pharmacological, and electrophysiological approaches, the present study explores the contribution of T cells to the pharmacological actions of A₃AR agonists in traumatic nerve injury–induced neuropathic pain.

Results and Discussion

Mouse sciatic nerve chronic constriction injury (CCI) leads to neuropathic pain (mechano-allodynia) that is maximal by day 7 (D7) and maintained for several weeks after injury (13). Intrapertoneal injection of highly selective A₃AR agonist MRS5980 at time of peak neuropathic pain reverses mechano-allodynia in both female and male mice (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI139299DSI), with effects lost in mice

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Conflict of Interest: DS is founder of BioIntervene Inc., a company that is developing A₃AR agonists for clinical use.
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Deficient in T and B cells (Rag-KO mice) (Figure 1A and Supplemental Figure 4). No significant difference in mechano-allodynia between WT and Rag-KO mice after nerve injury was observed, confirming previous studies (14). A3AR agonist doses were chosen from our previous studies to cause a near-to-maximal reversal of mechano-allodynia in this model (15). Adoptive transfer (D7 after CCI) of CD3+ T cells from WT mice fully reversed allodynia in this model (15). Adoptive transfer from A3AR knockout (Rag-KO) mice restored the anti-allodynic effects of MRS5980. Adoptive transfer of CD4+ T cells from Adora3-KO mice (E, n = 4) failed to restore the anti-allodynic effect of MRS5980 lost in both male and female Rag-KO mice after adoptive transfer of CD4+ T cells from Rag-KO mice (H, n = 9). Data are mean ± SD (A-H). *P < 0.05 vs. D0; †P < 0.05 vs D8/BL by 2-way repeated measures ANOVA (A and F) or 1-way ANOVA with Dunnett’s pair-wise comparisons (B-E, G, and H). ‡P < 0.05 vs. WT by 2-way repeated measures ANOVA with Sidak (A) or Tukey’s (F) pair-wise comparisons.

These results suggest that, following A3AR activation, CD4+ T cells release mediators that rapidly reverse allodynia. Therefore, we focused on IL-10, which can be released by T cells (16) and is able to reverse neuropathic pain states (17, 18). Moreover, neurons as well as both CD4+ T cells and CD8+ T cells express A3AR, IL-10, and IL-10R (19, 20). The anti-allodynic effects exerted by A3AR agonists were lost in IL10-KO and in IL-10 receptor (Il10r-KO) mice (Figure 1F and Supplemental Figure 1). Thus, an intact IL-10/IL-10R system is required for A3AR agonist effect (5). In order to test whether CD4+ T cells are a source of IL-10, we examined A3AR agonist responses in Rag-KO mice that were adoptively transferred with CD4+ T cells from Rag-KO mice. In both male and female Rag-KO mice repopulated with CD4+ T cells from IL10-KO mice, A3AR agonists failed to reverse mechano-allodynia (Figure 1G and Supplemental Figure 1), establishing CD4+ T cells as the predominant IL-10 source. In contrast, the A3AR agonist anti-allodynic effects were uncompromised in Rag-KO mice reconstituted with CD4+ T cells from Il10r-KO mice (Figure 1H and Supplemental Figure 1). Collectively, the data suggest that CD4+ T cell–derived IL-10, but not the presence of IL-10 receptor on the CD4+ cells, is necessary for the effects of A3AR agonists.

In Il10-KO mice, adoptive transfer of CD4+ T cells from WT but not Il10-KO mice restored the anti-allodynic effects of A3AR agonists (Figure 2, A–C and Supplemental Figure 1). This information supports similar findings in Rag-KO mice and the premise that CD4+ T cell–derived IL-10 is necessary for A3AR agonist effects.
A3AR effects lost in Il10r-KO mice are not restored by adoptive transfer of WT CD4+ T cells (Figure 2, D and E and Supplemental Figure 1), reinforcing the notion that CD4+ T cell–derived IL-10 is essential in A3AR agonists’ mode of action.

To determine whether A3AR activation on CD4+ T cells is required and sufficient for the IL-10 response, behavioral outcomes in Adora3-KO mice were investigated. A3AR agonists did not reverse mechano-allodynia in Adora3-KO mice (Figure 2F and Supplemental Figure 1). However, adoptive transfer of CD4+ T cells from WT donors but not from IL-10–KO mice into Adora3-KO mice completely restored the agonists’ anti-allodynic effects (Figure 2G and H and Supplemental Figure 1). These results establish that A3AR activation on CD4+ T cells drives the IL-10 response. As previously described (5, 21, 22), we observed no reduction of mechano-allodynia in Il10r-KO mice compared with WT mice. Moreover, the anti-allodynic responses to morphine were not altered in Il10r-KO (23) and Adora3-KO (24) mice compared with WT mice. No changes in contralateral paws were observed in any study (Supplemental Figures 3 and 4).

The hypersensitivity of primary sensory neurons that develops in the DRG is critically important in neuropathic pain development (25), and increased phosphorylation of GluN2B-containing N-methyl-D-aspartate receptors (NMDARs) at Tyr1472 [GluN2B(Tyr1472)] contributes to this increase (26, 27). Our data suggest that CD4+ T cell infiltration in the DRG attenuates neuronal excitability following A3AR activation. A- and C-type DRG neurons express the IL-10 receptor (alpha subunit, IL-10RA) (28). IL-10 can block phosphorylation of NMDARs by attenuating NMDA-induced intracellular calcium concentration increases (29), inhibiting protein kinases and phosphatases known to regulate NMDAR channel activity (30), inhibiting DRG neuronal firing (28, 31), and reducing neuronal firing indirectly by have effects on non-neuronal cells (11). Consistently, application of IL-10 to DRG neurons isolated from naive mice prevented action potential (AP) initiation (Figure 3, A–C, Supplemental Figure 5). Of note, DRG neurons exposed to IL-10 were still able to respond to the transient receptor potential vanilloid 1 (TPV1) agonist capsaicin (Supplemental Figure 6).
reduced neuronal firing in rat DRG neurons (35). This difference is possibly due to the reported lack of A3AR expression in mouse DRG neurons (36, 37).

When mouse DRG neurons were cultured in the absence of CD4+ T cells (Figure 4L), cocultured with CD4+ T cells (Figure 4C), cocultured with CD4+ T cells with anti-IL-10 antibody present (Figure 4F), or cocultured with CD8+ T cells (Figure 4I), the number of APs elicited by the current ramp in control conditions (before MRS5980 application) was similar among the groups. Results were replicated in DRG and CD4+ T cells isolated from CCI animals on D7 (Supplemental Figure 8 and Supplemental Table 1). Of note, DRG neurons isolated from CCI mice presented a markedly smaller current threshold to first AP (Supplemental Table 1), so ramp current injection was lowered to 15 pA to avoid signal saturation (Supplemental Figure 8).

Collectively, these results suggested a model whereby A3AR agonists reverse established hypersensitivity by activating A3AR expressed on CD4+ T cells to release IL-10, reducing neuronal DRG excitability (Graphical Abstract).

Methods
Detailed experimental methods are included with the Supplemental Material.
Figure 4. IL-10 released by CD4+ T cells is required for A3AR agonist–mediated inhibition of AP firing in cocultured mouse DRG neurons isolated from naive mice. Original current-clamp traces recorded by whole-cell patch-clamp technique in typical mouse DRG neurons. AP firing was evoked by a depolarizing ramp current injection (1 second; 30 pA; lower inset) once every 30 seconds. The A3AR agonist MRS5980 (300 nM) was applied in (A) DRG-CD4+ T cell cocultures, (B) DRG-CD4+ T cell cocultures in the presence of anti-IL-10 antibody (Ab IL-10; 0.5 μg/ml), (G) DRG-CD8+ T cell cocultures, and (J) DRG cultures. The number of APs elicited by the current ramp was plotted as a function of time in 4 different representative cells (B, E, H, and K) or was expressed as pooled data (mean ± SEM) in the bar graphs (L, n = 6; C, n = 10; F, n = 11; I, n = 7). Dotted gray lines indicate the 0 mV level. *P = 0.0120, paired Student’s t test. The number of APs elicited before MRS5980 application (with bars, ctrl) was not different in DRG neurons cultured alone (L), DRG neurons cocultured with CD4+ T cells (C), DRG neurons cocultured with CD4+ T cells in the presence of anti-IL-10 antibody (F), or DRG neurons cocultured with CD8+ T cells (I). One-way ANOVA with Bonferroni comparison: L vs. C, P = 0.3981; C vs. F, P = 0.1034; L vs. F, P > 0.9999; L vs. I, P > 0.9999. Scale bars: 300 ms; 50 mV.

Study approval. All animal procedures followed NIH guidelines and European Economic Community (86/609/CEE) recommendations. Experiments were approved by the Saint Louis University IACUC and by the University of Florence Animal Ethical and Care Committee.

Author contributions
DS conceived and designed the studies. EC designed the electrophysiology studies. MD, FL, SS, LDCM, CG, GK, CX, CW, LAG, FC, and EC performed the experiment and analysis. DKT, MLR, and KAJ provided key reagents. AO and DH provided technical input. DS, MD, SS, and FL prepared the manuscript with input from all authors. The order of the first co–authors was determined by degree of involvement with the project; MD and SS were involved during the project’s pilot phase; FL joined during the project’s maturation.

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