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The A_3 adenosine receptor (A_3AR) has emerged as a therapeutic target with A_3AR agonists to tackle the global challenge of neuropathic pain; investigation into their mode of action is essential for ongoing clinical development. A_3ARs on immune cells, and their activation during pathology, modulates cytokine release. Thus, immune cells as a cellular substrate for the pharmacological action of A_3AR agonists is enticing but unknown. Studies herein discovered that *Rag*^{KO} mice lacking T- and B-cells are insensitive to the anti-allodynic effects of A_3AR agonists versus wild-type (WT) mice. Similar findings were observed in interleukin-10 and interleukin-10 receptor knockout mice. Adoptive transfer of CD4$^+$ T-cells (CD4$^+$-T) from WT mice infiltrated the dorsal root ganglion (DRG) and restored A_3AR agonist-mediated anti-allodynia in *Rag*^{KO} mice; CD4$^+$-T from *Adora3*^{KO} or *Il10*^{KO} mice did not. Transfer of CD4$^+$-T from WT, but not *Il10*^{KO}, into *Il10*^{KO} mice fully reinstated anti-allodynic effects of A_3AR activation. Transfer of CD4$^+$-T from WT, but not *Il10*^{KO}, into *Adora3*^{KO} mice fully reinstated anti-allodynic effects of A_3AR activation. Notably, A_3AR agonism reduced DRG neuron excitability when co-cultured with CD4$^+$-T in an IL-10-dependent manner. A_3AR actions on CD4$^+$-T infiltrate 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 A_3AR on CD4$^+$-T cells to release of IL-10 is required and […]

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Disclosure of Potential Conflicts of Interest: Dr. Salvemini is founder of BioIntervene, Inc., a company developing A_3AR agonists for clinical use. All other authors have declared that no conflict of interest exist.

Abstract

The A_3 adenosine receptor (A_3AR) has emerged as a therapeutic target with A_3AR agonists to tackle the global challenge of neuropathic pain; investigation into their mode of action is essential for ongoing clinical development. A_3ARs on immune cells, and their activation during pathology, modulates cytokine release. Thus, immune cells as a cellular substrate for the pharmacological action of A_3AR agonists is enticing but unknown. Studies herein discovered that *Rag*^{KO} mice lacking T- and B-cells are insensitive to the anti-allodynic effects of A_3AR agonists versus wild-type (WT) mice. Similar findings were observed in interleukin-10 and interleukin-10 receptor knockout mice. Adoptive transfer of $CD4^+$ T-cells ($CD4^+$-T) from WT mice infiltrated the dorsal root ganglion (DRG) and restored A_3AR agonist-mediated anti-allodynia in *Rag*^{KO} mice; $CD4^+$-T from *Adora3*^{KO} or *Il10*^{KO} mice did not. Transfer of $CD4^+$-T from WT, but not *Il10*^{KO}, into *Il10*^{KO} mice fully reinstated anti-allodynic effects of A_3AR activation. Transfer of $CD4^+$-T from WT, but not *Il10*^{KO}, into *Adora3*^{KO} mice fully reinstated anti-allodynic effects of A_3AR activation. Notably, A_3AR agonism reduced DRG neuron excitability when co-cultured with $CD4^+$-T in an IL-10-dependent manner. A_3AR actions on $CD4^+$-T infiltrate 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 A_3AR on $CD4^+$-T cells to release of IL-10 is required and sufficient for A_3AR agonists as therapeutics.

Introduction

Chronic neuropathic pain (1) constitutes a large unmet medical need affecting 15-30 million people in the United States; 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 non-narcotic analgesics are needed. Recently, the Gi-coupled A_3 adenosine receptor (A_3AR) was identified as a novel target for therapeutic intervention with selective A_3AR agonists (4-6). Continued investigation into their mode of action is essential as these are in clinical development. Human and rodent immune cells, and in particular T cells including $CD4^+$ and $CD8^+$, express high A_3AR levels (7), but whether these receptors play a role in the beneficial agonist effects in neuropathic pain is unknown. Interestingly, A_3AR activation on circulating immune cells harvested from animal models of autoimmune disorders block the formation of neuroexcitatory/inflammatory cytokines such as TNF and interleukin 1β and enhance 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 (11) with documented positive effects in mitigating neuropathic pain (12). These data, in parallel fields of studies, point to a potential link between immune cells and IL-10 in A_3AR agonists' action. Using behavioral, genetic, pharmacological and electrophysiological approaches, studies herein explore the contribution of T cells in the pharmacological actions of A_3AR 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). Intraperitoneal injection of highly selective A_3AR agonist MRS5980 at time of peak neuropathic pain reverses mechano-allodynia in both female and male mice (**Figs. 1A, S1**) with effects lost in mice deficient in T and B cells (*Rag*^{KO} mice, **Figs. 1A, S4**). No significant difference in mechano-allodynia between WT and *Rag*^{KO} mice post nerve injury was observed confirming previous studies (14). A_3AR 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 from wild type (WT) mice restored the A_3AR agonist effects in *Rag*^{KO} mice **(Figs. 1B, S1)**. CD8$^+$-T adoptive transfer from WT mice did not restore A_3AR agonist anti-allodynic effects in *Rag*$^{-/-}$ mice; in contrast, adoptive transfer of CD4$^+$-T fully reinstated anti-allodynic effects in both male and female *Rag*$^{-/-}$ mice **(Figs. 1C,D, S1).** CD4$^+$-T adoptive transfer from A_3AR knockout (*Adora3*^{KO}) mice failed to restore the anti-allodynic effects of A_3AR agonists in *Rag*^{KO} mice indicating that A_3AR activation on CD4$^+$-T is required for A_3AR agonist anti-allodynic activity **(Figs. 1E, S1).** The anti-allodynic responses to morphine were unaltered in *Rag*^{KO} mice compared to WT (**Fig. S2**), which confirm that a lack of anti-allodynic responses is not a general, non-specific response.

These results suggest that CD4$^+$-T in response to A_3AR activation release mediators that can rapidly reverse allodynia. Therefore, we focused on interleukin-10 (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 and CD8$^+$-T express A_3AR, IL10 and IL10R (19, 20). The anti-allodynic effects exerted by A_3AR agonists were lost in *Il10*^{KO} and in IL-10 receptor mice (*Il10r*^{KO}) (**Figs. 1F, S1**). Thus, an intact IL10/IL10R system is required for A_3AR agonist effect (5). In order to test whether CD4$^+$-T are a source of this IL-10, we examined A_3AR agonist responses in *Rag*^{KO} mice that were adoptively transferred with CD4$^+$-T from *Il10*^{KO} mice. In both male and female *Rag*^{KO} mice repopulated with CD4$^+$-T from *Il10*^{KO} mice, A_3AR agonists failed to reverse mechano-allodynia (**Figs. 1G, S1**), establishing CD4$^+$-T as the predominant IL-10 source. In contrast, the A_3AR agonist anti-allodynic effects are uncompromised in *Rag*^{KO} mice reconstituted with CD4$^+$-T cells from *Il10r*^{KO} mice **(Figs. 1H, S1).** Collectively, the data suggest that CD4$^+$-T cell-derived IL10, but not the presence of IL10 receptor on the CD4$^+$ cells, is necessary for the effects of A_3AR agonists.

In *Il10*^{KO} mice, adoptive transfer of CD4$^+$-T cells from WT but not *Il10*^{KO} mice restored the anti-allodynic effects of A_3AR agonists **(Figs 2A-C, S1).** These findings support findings in *Rag*^{KO} mice and the premise that CD4$^+$-T cell-derived IL10 is necessary for A_3AR agonist effects.

A_3AR effects lost in *Il10r*^{KO} mice are not restored by adoptive transfer of WT CD4$^+$-T cells **(Figs. 2D-E, S1)** reinforcing the notion that CD4$^+$-T cell-derived IL10 is essential in A_3AR agonists' mode of action.

To determine whether A_3AR activation on CD4$^+$-T is required and sufficient for the IL-10 response, behavioural outcomes in *Adora3*^{KO} mice were investigated. A_3AR agonists did not reverse mechano-allodynia in *Adora3*^{KO} mice (**Figs. 2F, S1**). However, adoptive transfer of CD4$^+$-T from WT donors but not from IL-10^{KO} mice into *Adora3*^{KO} mice completely restored the agonists' anti-allodynic effects (**Figs. 2G-H, S1**). These results establish that A_3AR activation on CD4$^+$-T drives the IL-10 response. As previously described (5, 21, 22), we observed no reduction of mechanical allodynia in *Il10*^{KO} and *Il10r*^{KO} compared to WT mice; moreover, the anti-allodynic responses to morphine were not altered in *Il10*^{KO} (23) and *Adora3*^{KO} (24) mice compared to WT. No changes in contralateral paws were observed in any study **(Fig. S3-4).**

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 (NMDAR) at Tyr1472 [GluN2B(Tyr1472)] contributes to this increase (26, 27). Our data suggest that CD4$^+$-T infiltration in the DRG attenuates neuronal excitability following A_3AR activation. A- and C-type DRG neurons express the IL-10 receptor (alpha subunit, IL-10RA) (28). IL-10 can block phosphorylation of NMDAR 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 naïve mice prevented action potential (AP) initiation (**Figs. 3A-C, S5**). Of note, DRG neurons exposed to IL-10 were still able to respond to the transient receptor potential vanilloid 1 (TPV1) agonist capsaicin (**Fig. S6**).

Studies next examined whether A_3AR agonism leads to inhibition of DRG neuronal excitability via IL10 release from CD4$^+$-T cells. Immunofluorescence analysis of DRGs harvested from *Rag*^{KO} mice following adoptive transfer of CD4$^+$-T from WT mice expressing enhanced green fluorescent protein (GFP) showed increased CD4$^+$ T cell numbers in DRG ipsilateral to nerve injury compared to contralateral (**Fig. 3D,E**). The A_{2A}AR receptor subtype, not the A_3AR, seems to have the predominant role in lymphocytes migration (32, 33). Furthermore, in C57BL/6 mice, it has been reported that the absence of IL-10 receptor on the CD4$^+$ T cell surface does not impair trafficking in inflammatory conditions, suggesting IL-10/IL10R system as nonessential for T cell migration (34). So, although A_3AR activation of IL10 inhibition may affect T cell migration, we consider this to be unlikely. Intraperitoneal injection of MRS5980 caused a significant decrease in GluN2B(Tyr1472) phosphorylation in DRG ipsilateral to nerve injury in *Rag*^{KO}

mice after $CD4^+$-T adoptive transfer from WT mice compared to *Rag*^{KO} mice with no adoptive transfer (**Fig. 3F**).

To explore potential cross-talk between $CD4^+$-T and neurons in the DRG, we performed an *in vitro* study, co-culturing primary mouse DRG neurons with primary mouse $CD4^+$-T both cell types isolated from naïve animals. A_3AR agonist MRS5980 significantly decreased the number of APs evoked by a 30 pA ramp current in DRG neurons when co-cultured with $CD4^+$-T (**Fig. 4A-C**). Concurrently, a significant increase in current threshold (**Table S1**) was detected. These effects were prevented by an anti-IL-10 antibody (**Fig. 4D-F**) but not by a control IgG isotype (**Fig. S7**) and were not observed when DRG neurons were co-cultured with $CD8^+$-T (**Fig. 4G-I**). MRS5980 did not alter cell excitability when $CD4^+$-T were absent in the DRG culture (**Fig. 4J-L**). This result is at variance with findings that we have recently published, in which A_3AR activation reducted neuronal firing in rat DRG neurons (35). This difference is possibly due to the reported lack of A_3AR expression in mouse DRG (36, 37). When mouse DRG neurons were cultured in the absence of $CD4^+$-T (**Fig. 4L**), co-cultured with $CD4^+$-T (**Fig. 4C**), co-cultured with $CD4^+$-T with anti-IL-10 antibody present (**Fig. 4F**) or co-cultured with $CD8^+$-T (**Fig. 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 isolated from CCI animals on day 7 (**Fig. S8**, **Table S1**). Of note, DRG neurons isolated from CCI mice presented a significantly smaller current threshold to first AP (**Table S1**), so ramp current injection was lowered to 15 pA to avoid signal saturation (**Fig. S8**).

Collectively, these results suggested a model whereby A_3AR agonists reverse established hypersensitivity by activating A_3AR expressed on $CD4^+$-T to release IL-10, reducing neuronal DRG excitability (**Graphical Abstract**).

Methods

Detailed experimental methods are included with the supplemental materials.

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, GK, CX, CW, LAG, FC, EC performed the experiment and analysis, DKT, MLR, KAJ provided key reagents. AD and OH provided technical input. DS, MD, SS and FL prepared the manuscript with input from all authors.
Order of co-authorship: MD and SS involved during the project's pilot phase; FL joined during the project's maturation.

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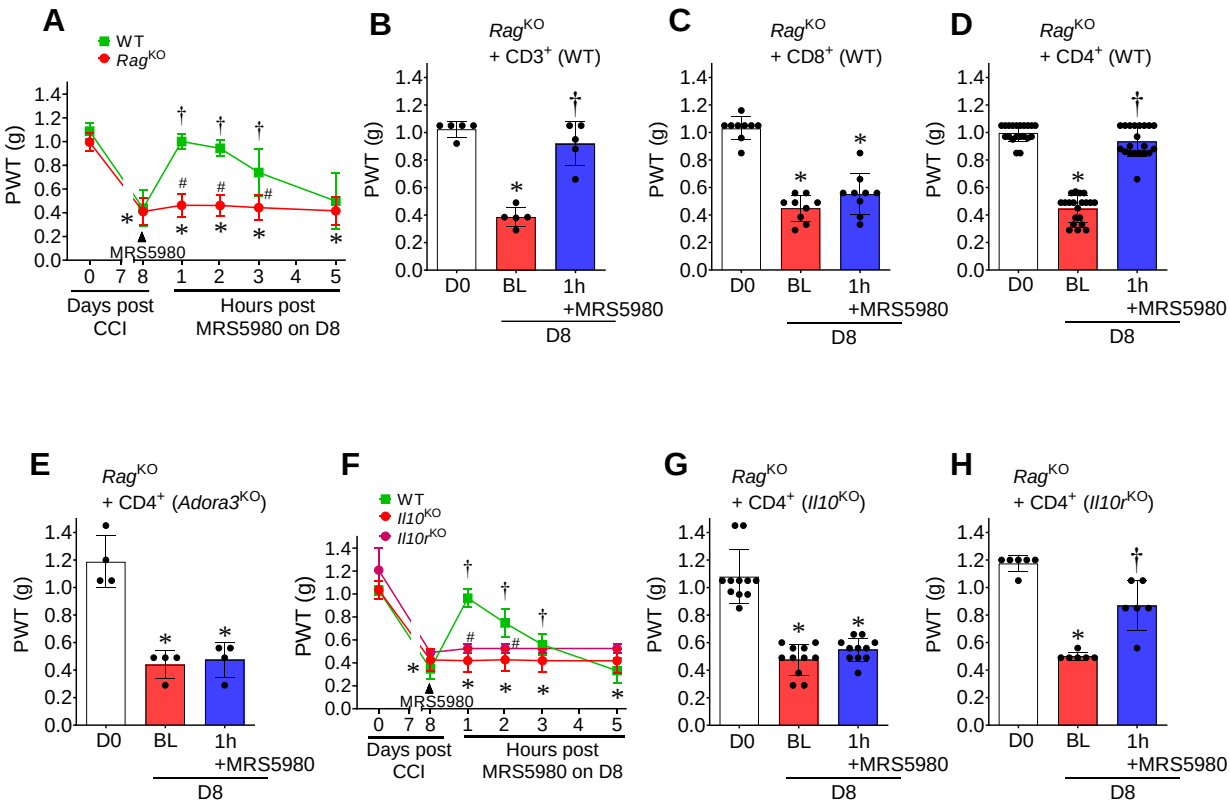

Figure 1. Anti-allodynic effects of A_3AR agonists require CD4$^+$-T. Injection of MRS5980 (1 mg/kg, i.p.) given at time of peak neuropathic pain reversed allodynia in male and female WT (n=7 males and n=5 females) but not *Rag*^{KO} (n=6 males and n=5 females) mice (**A**). Adoptive transfer of CD3$^+$ (**B,** n=5) or CD4$^+$ (**D**, n=15 males and n=5 females) T cells but not CD8$^+$-T (**C**, n=9) from WT mice into *Rag*^{KO} mice restored the anti-allodynic effects of MRS5980. Adoptive transfer of CD4$^+$-T from *Adora3*^{KO} mice (**E**, n=4) failed to restore the anti-allodynic effect of MRS5980. Injection of MRS5980 (1 mg/kg, i.p.), ipsilateral to nerve injury, during peak mechano-allodynia reversed allodynia in WT (n=5) but not in *Il10*^{KO} (n=5) and *Il10r*^{KO} (n=6) mice (**F**). The anti-allodynic effect of MRS5980 lost in both male and female *Rag*^{KO} after adoptive transfer of CD4$^+$-T from *Il10*^{KO} mice (**G**, n=6 males and n=5 females) was restored after adoptive transfer of CD4$^+$-T from *Il10r*^{KO} mice (**H**, n=9). Data are mean±SD (**A-H**) for n mice; *, p<0.05 vs D0; †, p<0.05 vs D8/BL by (**A,F**) two-way repeated measures ANOVA or (**B-E, G, H**) one-way ANOVA with Dunnett's pair-wise comparisons. #, p<0.05 vs WT by two-way repeated measures ANOVA with Sidak (**A**) or Tukey (**F**)'s pair-wise comparisons.

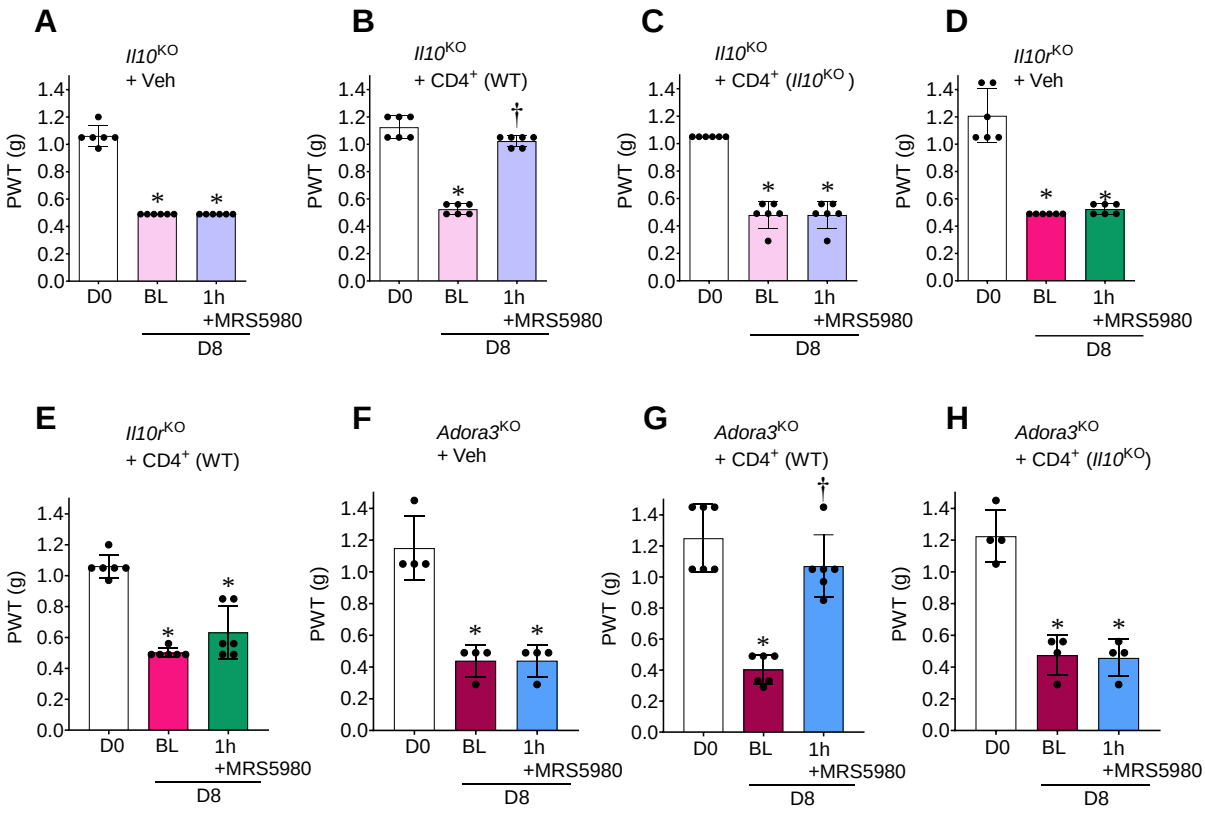

Figure 2. Activation of A_3AR expressed on CD4$^+$-T is required in the anti-allodynic effects of A_3AR agonist; role of IL-10. The anti-allodynic effect of MRS5980 were lost in *Il10*^{KO} (**A**, n=6) and *Il10r*^{KO} (**D**, n=6) mice. CD4$^+$-T adoptive transfer from WT (**B**, n=6) but not from *Il10* ^{KO} (**C**, n=6) mice restored MRS5980's anti-allodynic effects in *Il10* ^{KO} mice. Conversely, adoptive transfer of CD4$^+$-T from WT (**E**, n=6) mice did not restore the MRS5980's anti-allodynic effects in *Il10r* ^{KO} mice. The anti-allodynic effect of MRS5980 were lost in *Adora3*^{KO} mice (**F**, n=4). Adoptive transfer of CD4$^+$-T from WT (**G**, n=6) but not from *Il10*^{KO} (**H**, n=4) mice restored MRS5980's anti-allodynic effects in *Adora3*^{KO} mice. Data are mean±SD for n mice; *, p<0.05 vs D0; †, p<0.05 vs D8/BL by one-way ANOVA with Dunnett's pair-wise comparisons.

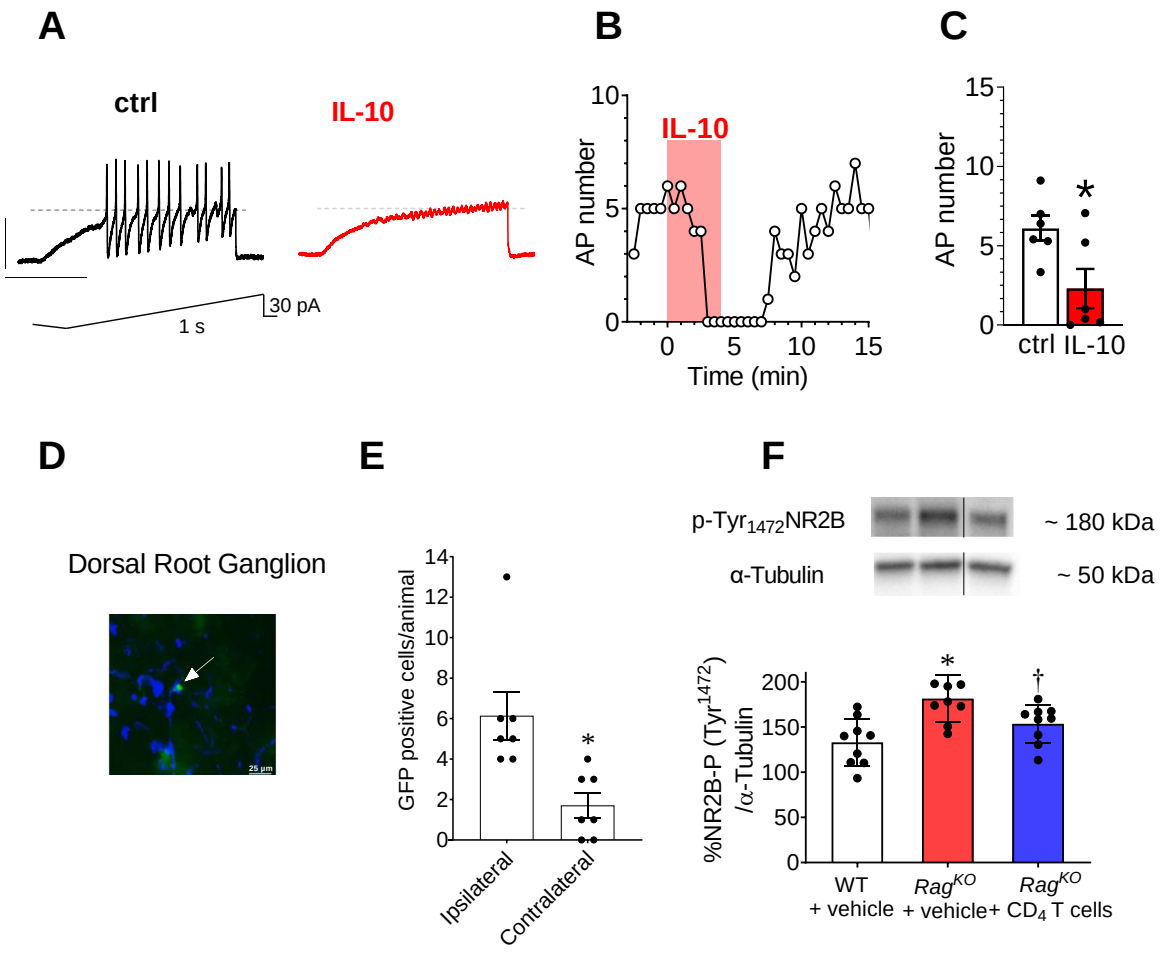

Figure 3. Functional effects of IL-10 on cell firing in DRG neurons and CD4⁺-T infiltration in mouse DRG. Original current-clamp traces recorded by whole-cell patch-clamp technique in a typical naïve mouse DRG neuron where IL-10 (0.5 µg/ml) reversibly inhibits AP firing evoked by a depolarizing ramp current injection (1 s; 30 pA: lower inset) once every 30 s (**A**). Dotted lines indicate the 0 mV level. The number of APs elicited by the current ramp was plotted as a function of time in the same cell (**B**) or was expressed as pooled data (mean±SEM) in the bar graph (**C**, n=6). *, p=0.0018, paired Student's *t*-test; Scale bars: 300 ms; 50 mV (**C**). CD4⁺-T (arrow) (magnification 40x) are present in the ipsilateral DRG of the *Rag*^{KO}mice reconstituted with CD4 T cells from WT GFP mice (green, GFP; blue, DAPI) (**D,E**, n=7). MRS5980 reduced Tyr1472 phosphorylation of GluN2B in the DRG of *Rag*^{KO} mice after adoptive transfer of CD4 T cells from WT mice (**F**, n=9). Density of each p-Tyr$_{1472}$GluN2B band was calculated relative to α-tubulin. Data are mean±SEM (**E**) or mean±SD (**F**) for n mice; *, p<WT+veh or Ipsilateral; †, p<0.05 vs *Rag*^{KO}+Veh by two-tailed Student's t-test (**E**) or (**F**) one-way ANOVA with Dunnett's pair-wise comparisons.

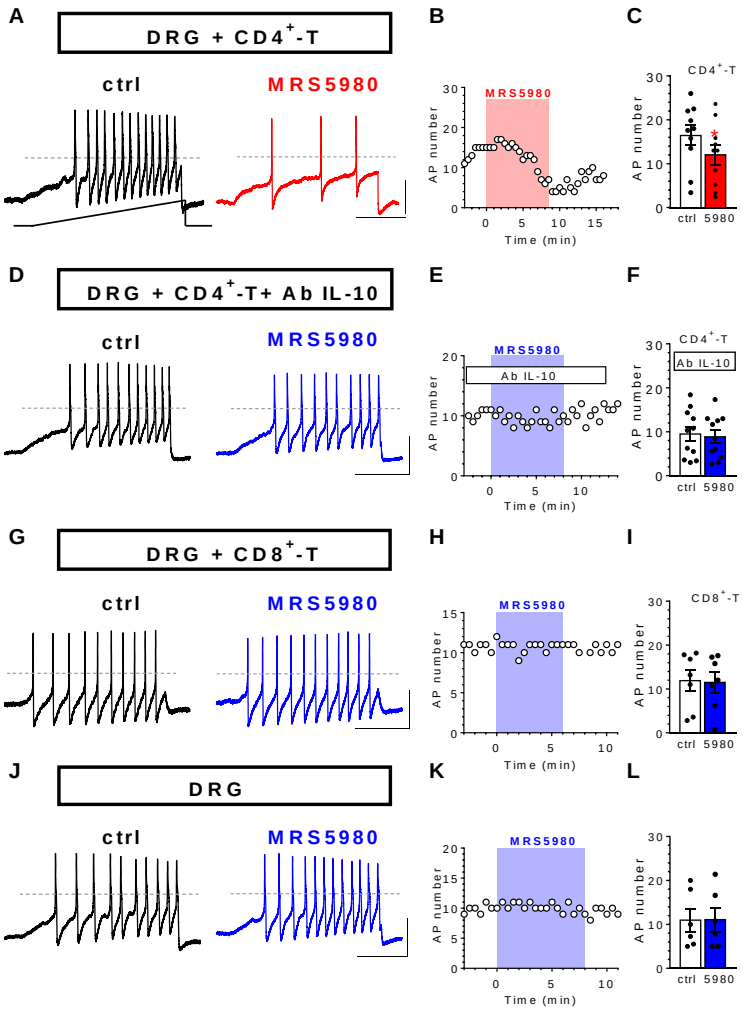

Figure 4. IL-10 released by CD4⁺-T is required for A_3AR agonist-mediated inhibition of AP firing in co-cultured mouse DRG neurons isolated from naïve mice. Original current-clamp traces recorded by whole-cell patch-clamp technique in typical mouse DRG neurons. AP firing was evoked by a depolarizing ramp current injection (1 s; 30 pA: lower inset) once every 30 s. The A_3AR agonist MRS5980 (300 nM) was applied in DRG-CD4⁺-T- co-cultures (**A**), in DRG-CD4⁺-T co-cultures in the presence of anti-IL-10 antibody (Ab IL-10; 0.5 µg/ml), (**D**) in DRG-CD8⁺-T co-cultures (**G**) and in DRG cultures (**J**). The number of APs elicited by the current ramp was plotted as a function of time in four different representative cells (**B**,**E**,**H,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 grey 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 co-cultured with CD4⁺-T (**C**), DRG neurons co-cultured with CD4⁺-T in the presence of anti-IL-10 antibody (**F**) or DRG neurons co-cultured with CD8⁺-T (**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.