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Cisplatin educates CD8+ T cells to prevent and resolve chemotherapy-induced peripheral neuropathy in mice

Geoffroy Laumet, **Jules D. Edralin**, **Robert Dantzer**, **Cobi J. Heijnen**, and **Annemieke Kavelaars**

Laboratory of Neuroimmunology, Department of Symptom Research, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit 384, Houston, TX, 77030

Abstract

The mechanisms responsible for the persistence of chemotherapy-induced peripheral neuropathy (CIPN) in a significant proportion of cancer survivors are still unknown. Our previous findings show that $CD8^+$ T cells are necessary for the resolution of paclitaxel-induced mechanical allodynia in male mice. In the present study, we demonstrate that $CD8^+$ T cells are not only essential for resolving cisplatin-induced mechanical allodynia, but also to normalize spontaneous pain, numbness, and the reduction in intra-epidermal nerve fiber density in male and female mice. Resolution of CIPN was not observed in $Rag2^{-/-}$ mice that lack T and B cells. Reconstitution of $Rag2^{-/-}$ mice with $CD8^+$ T cells *prior* to cisplatin treatment normalized the resolution of CIPN. *In vivo* education of $CD8^+$ T cells by cisplatin was necessary to induce resolution of CIPN in $Rag2^{-/-}$ mice because adoptive transfer of $CD8^+$ T cells from naïve WT mice to $Rag2^{-/-}$ mice *after* completion of chemotherapy did not promote resolution of established CIPN. The $CD8^+$ T cell-dependent resolution of CIPN does not require epitope recognition by the T cell receptor (TCR). Moreover, adoptive transfer of cisplatin-educated $CD8^+$ T cells to $Rag2^{-/-}$ mice prevented CIPN development induced by either cisplatin or paclitaxel, indicating that the activity of the educated $CD8^+$ T is not cisplatin-specific.

In conclusion, resolution of CIPN requires *in vivo* education of $CD8^+$ T cells by exposure to cisplatin. Future studies should examine whether *ex vivo* $CD8^+$ T cell education could be applied as a therapeutic strategy for treating or preventing CIPN in patients.

Keywords

chemotherapy-induced peripheral neuropathy; cisplatin; $CD8^+$ T cells

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Correspondence: Dr. Annemieke Kavelaars, Laboratory of Neuroimmunology, Department of Symptom Research, The University of Texas MD Anderson Cancer Center, Unit 384, 1515 Holcombe Blvd., Houston, TX, 77030, USA. Tel 713-794-5297, fax 713-745-3475; AKavelaars@mdanderson.org; https://www.mdanderson.org/research/departments-labs-institutes/labs/neuroimmunology-laboratory.html.

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1. INTRODUCTION

Chronic pain affects between 11%–40% of Americans [28; 46]. Chronic pain results from abnormal activity of the neurons of the nociceptive pathway within the dorsal root ganglia (DRG) and the central nervous system [64]. Increasing evidence suggests a role for non-neuronal cells in chronic pain [27; 53; 69]. Notably, immune cells have been shown to be important regulators of the transition from acute to chronic pain [53]. Activated macrophages and microglia release pro-inflammatory factors which sensitize neurons in DRG and spinal cord leading to increased pain signaling [5; 68]. Less studied, but likely as important, is the role of anti-inflammatory macrophages, which promote resolution of pain [3; 15; 67]. The contribution of the adaptive immune system to chronic pain is less clear, and the role of T cells in chronic pain is especially debated. Studies have reported that depletion of T cells attenuates allodynia in response to peripheral nerve injury in rodents [10; 14]; in models of neuropathic pain induced by nerve injury or chemotherapy and in a model of inflammatory pain associated with rheumatoid arthritis, we and others have shown that the absence of T cells does not affect the onset of allodynia and even worsens the intensity or duration of allodynia [1; 2; 30; 36; 52; 61]. In addition, evidence suggests that the contribution of T cells to neuropathic and inflammatory pain may be sex-specific [58].

Most of the efforts to study the role of T cells in pain has been focused on $CD4^+$ T cells [1; 14; 61]. However, we demonstrated recently that $CD8^+$ T cells but not $CD4^+$ T cells are necessary for the resolution of mechanical allodynia in mice treated with paclitaxel [30]. In male *Rag1*$^{-/-}$ mice (lacking mature B and T cells), allodynia was significantly prolonged after paclitaxel treatment. Reconstitution of male *Rag1*$^{-/-}$ mice with $CD8^+$ T cells before paclitaxel treatment normalized resolution of allodynia.

Understanding the mechanisms underlying resolution of chemotherapy-induced peripheral neuropathy (CIPN) is important, because CIPN does not resolve after treatment cessation in 20%–30% of patients and can persist for years [45; 54]. To study the underlying mechanisms of persistent CIPN, we used a mouse model of CIPN induced by cisplatin, a platinum-based drug used to treat solid tumors [21; 44]. Our mouse model of CIPN shows spontaneous pain, mechanical allodynia and peripheral numbness [31; 39; 65; 70]. At the structural level, cisplatin-induced peripheral neuropathy is associated with a reduction in density of intra-epidermal nerve fibers (IENF) [31; 55].

Here, we first tested the hypothesis that $CD8^+$ T cells are essential for the resolution of all signs of CIPN in female and male mice treated with cisplatin. Second, we tested the hypothesis that $CD8^+$ T cells need to be educated by cisplatin in order to be capable of resolving CIPN.

2. METHODS

2.1. Animal model

Female and male (10–14 weeks old) offspring of wild type (WT), *Rag2*$^{-/-}$ (no mature T and B cells), *Ifng*$^{-/-}$, and OT-I (major histocompatibility complex class I-restricted, ovalbumin-specific $CD8^+$ T cell transgenic) mice in a C57BL/6J background obtained from Jackson

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Laboratory (Bar Harbor, Maine) were housed and bred at The University of Texas MD Anderson Cancer Center. All procedures were approved by the MD Anderson Animal Care and Use Committee. All protocols conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 86–23) and ARRIVE guidelines.

To induce CIPN, mice were treated intraperitoneally with cisplatin (Teva Pharmaceutical Industries Limited, North Wales, Pennsylvania) for 3 days at a dose of 2 mg/kg (cumulative dose 6 mg/kg) or paclitaxel on day 0 and 2 at a dose of 2 mg/kg. Phosphate-buffered saline (PBS) was used as vehicle. Animals were randomly assigned to the different treatment groups, and measures were taken by investigators blinded to treatment.

2.2. Adoptive transfer

Adoptive transfer of $CD8^+$ T cells was performed before or after chemotherapy injection, as previously described [30; 33]. Spleens were collected from WT, OT-I, or *Ifng*$^{-/-}$ mice, and single-cell suspensions were obtained by passing spleens though a 70-μm mesh. $CD8^+$ T cells were isolated using negative selection kit II (#130-095-130, Miltenyi Biotec Inc, San Diego, California). Three million $CD8^+$ T cells were intravenously (IV) injected into the tail vein in a 100-μL volume. Control mice received an IV injection of vehicle (PBS plus 0.1% bovine serum albumin). Homing and survival of the adoptively transferred cells were confirmed by flow cytometry and quantitative real-time polymerase chain reaction (qRT-PCR). Educated $CD8^+$ T cells were obtained when WT mice had recovered from CIPN, 8 weeks after 5 daily injections of cisplatin 2 mg/kg (cumulative dose 10 mg/kg).

2.3. Flow cytometry

Blood obtained by cardiac puncture was collected into heparinized tubes. Leukocytes were stained with anti-CD45-APC (#561018; BD Biosciences, San Jose, California) and T cells were labeled with anti-CD3-PE and anti-CD8a-FITC (#561799 and #553031; BD Biosciences). Red blood cells were lysed using lysing buffer (#555899; BD Biosciences) and samples were analyzed by flow cytometry (C6 Accuri; BD Biosciences) as described [30; 33].

2.4. Behavioral assessment

2.4.1. Mechanical allodynia—Mechanical allodynia was monitored using the von Frey method, as previously described [34; 35]. Mice were placed in plastic cages (10 × 10 × 10 cm) on a mesh stand (IITC Life Science, Woodland Hills, California) for 30 minutes for habituation. A series of calibrated filaments were applied to the plantar surface of the hind paw for up to 5 seconds. The "up and down" method was used to calculate the force needed for 50% likelihood of withdrawal [11]. The data presented represent group means of the average of both hind paws for each mouse.

2.4.2. Spontaneous pain—Spontaneous pain was measured using a conditioned place preference (CPP) paradigm [31; 62]. The CPP apparatus consists of 2 chambers (18 × 20 cm each, one dark, one bright) connected by a 15-cm hallway (Stoelting Co., Wood Dale, Illinois). On the first day, each mouse was placed in the hallway and permitted to freely explore the apparatus for 15 minutes. On the next 4 days in the morning, mice were injected

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intraperitoneally with PBS and 10 minutes later placed individually in the dark chamber for 15 minutes. Four hours later, each mouse received an analgesic dose of retigabine (10 mg/kg in PBS; #R-100; Alomone Labs, Jerusalem, Israel) administered intraperitoneally and was placed in the bright chamber. On the sixth day, mice were allowed to freely explore the apparatus for 15 minutes; the change in the amount of time spent in the bright (previously retigabine-paired) chamber was quantified. The preconditioning and post-conditioning test results were recorded and analyzed using Ethovision XT video tracking software (Noldus Information Technology Inc., Leesburg, Virginia).

2.4.3. Adhesive recognition test—

We used a modified version of the adhesive recognition test [6] to assess numbness. A 3/16” round sticker (Teeny Tough-Spot; USA Scientific Inc, Ocala, Florida) was placed on the plantar surface of the hind paw. The mouse was placed in a plastic box (10 × 20 × 10 cm) and was videotaped from below to determine the time until the mouse responds to the sticker, as previously described [31; 41].

2.5. Intra-epidermal nerve fiber staining

Intra-epidermal nerve fiber (IENF) density was quantified in glabrous skin on the plantar surface of the hind paws, as previously described [31; 55]. Briefly, frozen 25-μm sections were incubated with antibodies against the pan-neuronal marker PGP9.5 (rabbit; Bio-Rad AbD Serotec Limited, Oxford, United Kingdom) and Collagen IV (goat; Southern Biotech, Birmingham, Alabama) followed by Alexa-594 donkey anti-rabbit and Alexa-488 donkey anti-goat antibodies (Life Technologies, Carlsbad, California). IENF density was expressed as the number of nerve fibers crossing the basement membrane/length of the basement membrane (mm) and was measured using 4 mice/group and 5 random pictures/mouse.

2.6. Quantitative RT-PCR

Mice were terminated by CO_2 exposure and transcardially perfused with ice-cold PBS. Spleen and DRG were quickly harvested and snap-frozen in liquid nitrogen. RNA were isolated using the Trizol (Invitrogen, Carlsbad, California) chloroform method, and qRT-PCR was used to quantify mRNA levels using prime assay primers (Integrated DNA Technologies, Coralville, Iowa).

2.7. Statistical analysis

Differences in behavioral activity and expression levels were assessed by Student’s t-test, one-way or repeated-measure two-way ANOVA followed by Bonferroni correction for multiple tests, depending on experimental design. Significant difference are indicated in graphs as ***=P<0.001, **=P<0.01, *=P<0.05.

3. Results

3.1. CD8$^+$ T cells are necessary for resolution of CIPN

To evaluate the contribution of CD8$^+$ T cells to resolution of mechanical allodynia induced by cisplatin, we compared WT mice with *Rag2*$^{-/-}$ mice, which lack mature B and T cells. Mice were treated with 3 daily intraperitoneal injections of cisplatin. The onset and intensity of allodynia was similar in cisplatin-treated male and female WT and *Rag2*$^{-/-}$ mice.

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Resolution of allodynia was markedly delayed in *Rag2*$^{-/-}$ mice of both sexes (males: Figure 1A; females: Figure 1B). Adoptive transfer of naïve CD8^{+} T cells to *Rag2*$^{-/-}$ mice 10 days before cisplatin injection normalized the resolution of allodynia in both male and female mice (Figure 1). CD8^{+} T cell reconstitution in the *Rag2*$^{-/-}$ mice was confirmed by quantifying CD8^{+} T cells in peripheral blood by flow cytometric analysis, and gene-expression analysis of *Cd3e* and *Cd8a* in spleen and DRG (Supplemental Digital Content 1A,B shows results from the reconstitution of *Rag2*$^{-/-}$ mice with T cells). CD8^{+} T cells are one of the main producers of interferon (IFN)-γ [48]. We tested whether CD8^{+} T cells must be capable of producing IFN-γ in order to resolve cisplatin-induced allodynia. We found that resolution of allodynia was similar in *Rag2*$^{-/-}$ mice reconstituted with CD8^{+} T cells from *Ifng*$^{-/-}$ mice or WT mice, indicating that CD8^{+} T cells promote resolution of allodynia independent of their capability to produce IFN-γ (Figure 1C).

We previously showed that cisplatin induces spontaneous pain and numbness in WT mice [31]. To investigate whether spontaneous pain is prolonged in *Rag2*$^{-/-}$ mice, we used a CPP paradigm 3 weeks after cisplatin injection. At this time point, mechanical allodynia had already resolved in WT mice, whereas *Rag2*$^{-/-}$ mice still displayed allodynia (Figure 1). Three weeks after cisplatin, *Rag2*$^{-/-}$ mice developed a preference for the analgesic-paired chamber, whereas WT mice no longer did. *Rag2*$^{-/-}$ mice reconstituted with CD8^{+} T cells behaved like WT mice (Figure 2A). These findings indicate that CD8^{+} T cells are necessary and sufficient for the resolution of spontaneous pain after cisplatin treatment.

To assess cisplatin-induced peripheral numbness, we performed the adhesive removal test 3 weeks after cisplatin injection. We measured the time it took each mouse to respond to a small sticker placed on the plantar surface of the hind paw [6]. Cisplatin-treated *Rag2*$^{-/-}$ mice took approximately twice as long to respond to the sticker as compared to PBS-treated *Rag2*$^{-/-}$ mice, and PBS- or cisplatin-treated WT mice. *Rag2*$^{-/-}$ mice reconstituted with CD8^{+} T cells responded to the sticker as fast as cisplatin-treated or PBS-treated WT mice did (Figure 2B). These data indicate that CD8^{+} T cells are required for the resolution of numbness after cisplatin treatment.

Next, we determined the contribution of CD8^{+} T cells to normalization of IENF density in the hind paw after completion of cisplatin treatment. In WT mice cisplatin induces a reduction of IENF density as early as 8 days (Supplemental Digital Content 2 shows IENF density reduction at 8 days after cisplatin). Three weeks after cisplatin, IENF density had normalized in cisplatin-treated WT mice (Figure 2C,D). Three weeks after cisplatin, IENF density in *Rag2*$^{-/-}$ mice was still reduced in comparison to all other groups. Reconstitution of *Rag2*$^{-/-}$ mice with CD8^{+} T cells restored the IENF density to a similar level as that found in the WT mice at 3 weeks after cisplatin (Figures 2C and 2D). These data demonstrate that CD8^{+} T cells are critical for restoration of IENF density.

3.2. Resolution of established CIPN requires educated CD8^{+} T cells

To determine whether adoptive transfer of CD8^{+} T cells can reverse already established CIPN, we reconstituted *Rag2*$^{-/-}$ mice with CD8^{+} T cells 7 days after cisplatin injection (at the peak of allodynia). Adoptive transfer of CD8^{+} T cells from naïve mice into *Rag2*$^{-/-}$ mice after cisplatin injection did not reverse established allodynia (Figure 3A). This was not due

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to insufficient reconstitution of $CD8^+$ T cells, because the number of $CD8^+$ T cells was similar in *Rag2*$^{-/-}$ mice reconstituted with $CD8^+$ T cells before or after cisplatin (Supplemental Digital Content 1C, shows results from the reconstitution of *Rag2*$^{-/-}$ mice with T cells). Next, we tested whether $CD8^+$ T cells must be 'educated' by prior exposure to cisplatin to be capable of promoting resolution of established CIPN. For this purpose, WT mice were treated with 5 injections of 2 mg/kg cisplatin. Eight weeks after cisplatin injection, their $CD8^+$ T cells were harvested from spleens and adoptively transferred to cisplatin-treated-*Rag2*$^{-/-}$ mice (Figure 3B). Adoptive transfer of these educated $CD8^+$ T cells into *Rag2*$^{-/-}$ mice reversed established CIPN (Figure 3C). In addition, adoptive transfer of educated $CD8^+$ T cells, but not of naïve $CD8^+$ T cells, after completion of cisplatin treatment normalized IENF density as well (Figures 3D and 3E).

Education of $CD8^+$ T cells can involve antigen recognition by the T-cell receptor (TCR) complex. To assess whether the effect of educated $CD8^+$ cells was epitope-specific involving recognition by the TCR complex that recognizes only the irrelevant chicken ovalbumin antigen [24]. We reconstituted *Rag2*$^{-/-}$ mice with $CD8^+$ T cells from OT-I or WT mice. Resolution of allodynia was similar in female and male *Rag2*$^{-/-}$ mice reconstituted with $CD8^+$ T cells from WT or OT-I mice (Figures 4A and 4B). These data indicate that CIPN resolution is independent of specific epitope recognition by the TCR.

3.3. $CD8^+$ T cells educated *in vivo* can prevent CIPN

We next tested whether adoptive transfer of *in vivo* educated $CD8^+$ T cells by prior exposure of the donor mice to cisplatin can also prevent development of CIPN. $CD8^+$ T cells were educated by exposing WT mice to cisplatin or PBS for 5 days; 8 weeks after completion of treatment, $CD8^+$ T cells were collected from spleens and adoptively transferred to naïve *Rag2*$^{-/-}$ mice (Figure 5A). Ten days after reconstitution with $CD8^+$ T cells, *Rag2*$^{-/-}$ mice were treated with cisplatin or PBS. The development of CIPN was completely prevented in *Rag2*$^{-/-}$ mice reconstituted with educated $CD8^+$ T cells. This preventive effect of educated $CD8^+$ T cells was observed in both sexes (Figures 5B and 5C). Reconstitution of *Rag2*$^{-/-}$ mice with educated $CD8^+$ T cells also prevented cisplatin-induced reduction in IENF density (Figures 5D and 5E).

We then assessed whether the preventive effect of educated $CD8^+$ T cells was specific for resolution of cisplatin-induced neuropathy. $CD8^+$ T cells were educated by cisplatin and adoptively transferred to *Rag2*$^{-/-}$ mice as described above (Figure 5A). Ten days after reconstitution with $CD8^+$ T cells, *Rag2*$^{-/-}$ mice were treated with paclitaxel. Cisplatin-educated $CD8^+$ T cells were capable of preventing development of allodynia in both paclitaxel- and cisplatin-treated *Rag2*$^{-/-}$ acceptor mice. (Figure 5B,C,F).

4. DISCUSSION

We demonstrate that $CD8^+$ T cells are essential for resolution of all signs of cisplatin-induced peripheral neuropathy in both male and female mice. Our findings apply to allodynia, spontaneous pain, numbness, and reduction in IENF density in both sexes. All these signs of CIPN are significantly prolonged in T-cell–deficient *Rag2*$^{-/-}$ mice and are normalized in *Rag2*$^{-/-}$ mice reconstituted with $CD8^+$ T cells. These original findings

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strengthen the concept that resolution of CIPN depends on an active endogenous process mediated by CD8$^+$ T cells.

Importantly, we show for the first time that education of CD8$^+$ T cells by cisplatin is a critical step in the resolution of established CIPN. Furthermore, CD8$^+$ T cells educated *in vivo* by cisplatin are capable of preventing CIPN. Interestingly, cisplatin educated CD8$^+$ T cells not only resolve cisplatin-induced CIPN but also paclitaxel-induced CIPN. These findings indicate that the activity of the educated CD8$^+$ T is not cisplatin-specific and imply that *ex vivo* education of CD8$^+$ T cells might be a promising future approach to prevent or treat CIPN in cancer patients and survivors.

The contribution of T cells to chronic pain is controversial. In the spared nerve injury model of neuropathic pain, some authors reported that the onset of allodynia was attenuated in *Rag1*$^{-/-}$ mice [14]; others found no difference in the onset and intensity of allodynia between WT and *Rag1*$^{-/-}$ mice [52]. In CIPN models, we and others showed that T-cell–deficient mice or mice depleted from T cells have a similar onset of allodynia, compared with WT mice [30; 40]. In contrast, Liu et al. reported an exacerbation of paclitaxel-induced allodynia after transfer of CD8$^+$ T cells into WT mice [38]. A potential explanation for this discrepancy with our findings is that Liu et al. administered the T cells intrathecally; T cells are not present (or are at a very low level) in the spinal cords of control and neuropathic-pain animals [16; 20; 26]. We administered the (educated) CD8$^+$ T cells intravenously and these cells will end up first in the secondary lymphoid organs, allowing the T cells to differentiate and/or to regulate activity of other cell types such as macrophages. Subsequently, these macrophages may migrate from the spleen to DRG or spinal cord to suppress the pain response. Indeed, we and others have shown that macrophages produce IL-10 to promote resolution of inflammatory pain [3; 67].

Our findings are in line with accumulating evidence that T cells play a beneficial role after nerve injury and promote recovery after stroke in a mouse model [42]. It is interesting to note that a reduction in circulating CD8$^+$ T cells has been reported in patients suffering from complex regional pain syndrome and fibromyalgia [29]. This reduction in circulating CD8$^+$ T cells may be sufficient to account for their inability to promote resolution of pain or prevent development of chronic pain. A protective role for T cells has also been described in models of stress; T-cell–deficient mice are less resilient to stress-induced depression-like behavior [7; 37]. In addition, we recently showed that inflammation-induced depression-like behavior is prolonged in T cell-deficient mice [33]. These data point to a common role for T cells in endogenous resolution pathways in comorbid pain and depression.

In the present study, we did not observe differences in onset or severity of CIPN between male and female mice, while resolution tends to be slightly faster in male mice. Importantly, however, in both sexes CD8$^+$ T cells are required for resolution of CIPN. It has been suggested that neuropathic pain after nerve injury or inflammatory pain induced by lipopolysaccharide (LPS) is mostly T-cell dependent in female and microglia dependent in male mice [58]. In the present study, the absence of CD8$^+$ T cells prolonged cisplatin-induced allodynia to the same extent in female and male mice. Likewise, educated CD8$^+$ T cells prevented cisplatin-induced allodynia in both sexes. Unlike nerve injury or intrathecal

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injection of lipopolysaccharide, cisplatin does not induce activation of microglia [51; 57; 58; 71], which could explain the absence of sex differences as microglia are one of the main drivers of sex difference in the nervous system [63].

Even if resolution of all signs of CIPN requires $CD8^+$ T cells, it remains to be determined what the underlying neuroimmune mechanisms are. It is unlikely that the chemotherapy-induced loss in IENF density and pain are causally related and it may well be possible that their resolution mechanisms differ. With respect to resolution of pain, we examined the contribution of IFN-γ produced by $CD8^+$ T cells. IFN-γ activates GABAergic inhibitory currents [19] which are reduced in the spinal cord for CIPN [8; 9; 12]. However, our finding that $CD8^+$ T cells from *Ifng*$^{-/-}$ and WT mice both promote resolution of CIPN indicates that $CD8^+$ T cells do not need to produce IFN-γ to resolve CIPN. We also know that $CD8^+$ T cells themselves do not need to produce IL-4 or IL-10 to induce allodynia resolution [30]. However, we did show previously that IL-10 signaling is required for normalization of pain, indicating indirect effects of $CD8^+$ T cells through promoting IL-10 signaling. In addition, $CD8^+$ T cells may have direct or indirect neuroimmune interactions with neurons or release factors to promote normalization of IENF density during resolution of CIPN.

Interestingly, our data indicate that $CD8^+$ T cells need to be educated to develop the capacity to promote resolution of CIPN as only adoptive transfer of educated $CD8^+$ T cells reversed established CIPN. The resolution of CIPN does not require epitope recognition by the TCR complex as $CD8^+$ T cells from OT-I mice, that can only recognize and respond to ovalbumin-derived peptide antigens, were as effective to resolve CIPN as $CD8^+$ T cells from WT mice. A substantial population of $CD8^+$ T cells has been shown to express phenotypical markers of immunological memory in absence of antigen exposure [66]. These $CD8^+$ T cells develop a memory phenotype in response to TCR stimulation independently of epitope recognition [13; 18; 59] and stimulation by cytokines [23; 49; 50]. As IL-10 promotes the maturation of memory-like $CD8^+$ T cells and potentiates the activity of $CD8^+$ T cells [17; 22; 32; 47] it is possible that $CD8^+$ T cells are educated by IL-10 in secondary lymphoid organs toward a regulatory phenotype to promote resolution of CIPN. In accordance with this hypothesis we have already shown that IL-10 is necessary for CIPN resolution but that the $CD8^+$ T cells do not need to produce IL-10 [30]. In addition, cisplatin has immunogenic properties and might induce expression of co-stimulatory molecules on antigen-presenting cells to stimulate $CD8^+$ T cells to exert their regulatory effect [4; 60].

A salient finding of our work is that adoptive transfer of educated $CD8^+$ T cells to *Rag2*$^{-/-}$ mice prevented CIPN in recipient mice. The protective effects of the educated $CD8^+$ T cells studied here in the context of CIPN are reminiscent of previous observations indicating the capacity of "educated" T cells to transfer stress resilience or alleviate demyelination in a multiple sclerosis model. In these studies, adoptive transfer of lymphocytes from stressed WT mice conferred antidepressant effects on *Rag2*$^{-/-}$ mice [7] and adoptive transfer of $CD4^+$ T regulatory cells from experimental autoimmune encephalomyelitis (EAE) mice delayed the progression of EAE [43].

We demonstrated here that transfer of cisplatin-educated $CD8^+$ T cells prevent CIPN induced either with cisplatin or paclitaxel again confirming that there is no epitope

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specificity involved in the education of CD8+ T cells. Cisplatin and paclitaxel have different intracellular anticancer mechanisms: cisplatin induces DNA adducts and paclitaxel promotes stabilization of microtubules although the cellular outcome in peripheral sensory neurons may be similar and includes mitochondrial dysfunction, retraction of IENF, and neuronal hyperexcitability in both cases [39; 56]. Whether the preventive effects of cisplatin educated-CD8+ T cells would apply to all types of chemotherapeutic agents that induce CIPN, remains to be determined.

The educated CD8+ T cells used here were obtained 8 weeks after cisplatin injection; preliminary data indicate that they retain their protective capacity to prevent CIPN even when they are obtained 20 weeks after completion of cisplatin treatment (data not shown). These data suggest that the educated phenotype is long-lasting.

5. CONCLUSION

Our study documents two important findings. First, we show that resolution of CIPN after cisplatin treatment is an active process that includes a critical role for CD8+ T cells in the resolution of allodynia, spontaneous pain, numbness, and reduction of IENF density, regardless of sex. Moreover, CD8+ T cells need to be educated by cisplatin in order to resolve established CIPN. We also demonstrate that cisplatin educates CD8+ T cells which can be used to prevent CIPN induced either by cisplatin or paclitaxel. Our work strengthen the prospect for immune cell-based therapies in neurological disorders [25], such as *ex vivo* education of autologous peripheral CD8+ T cells, to relieve the burden of CIPN and restore quality of life of cancer survivors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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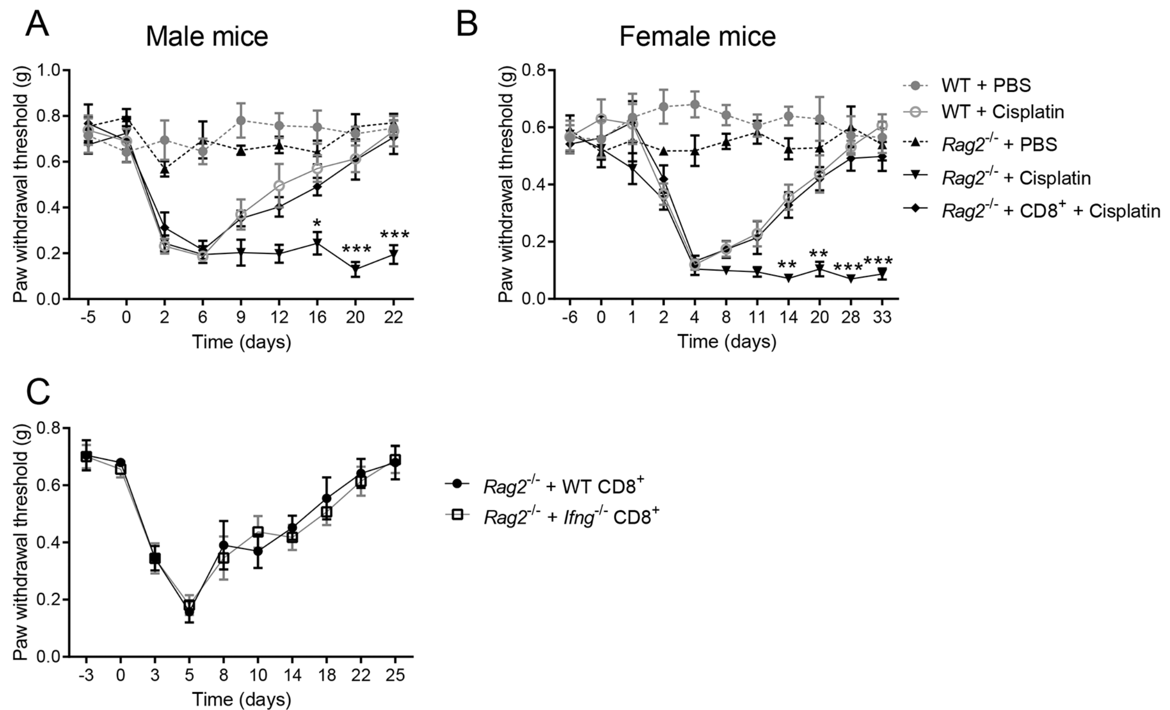

Figure 1. Effects of CD8$^+$ T cells on mechanical allodynia induced by cisplatin in female and male mice

Cisplatin (2 mg/kg) or PBS was administrated on days 0, 1, and 2 to male and female WT mice, *Rag2*$^{-/-}$ mice, and *Rag2*$^{-/-}$ mice reconstituted with CD8$^+$ T cells on day −10. **(A)** Mechanical sensitivity in male mice. Two-way repeated measures ANOVA interaction (time × genotype): $F(32,200)=5.20$, $P<0.0001$ (n=6 mice/group). **(B)** Mechanical sensitivity in female mice. Two-way repeated measures ANOVA interaction (time × genotype): $F(40,280)=6.95$, $P<0.0001$ (n=6 mice/group). ***$p<0.001$, **$p<0.01$, *$p<0.05$ between cisplatin-treated *Rag2*$^{-/-}$ vs. *Rag2*$^{-/-}$ mice reconstituted with CD8+ T cells". **(C)** Female *Rag2*$^{-/-}$ mice were reconstituted with CD8$^+$ T cells from WT or *Ifng*$^{-/-}$ mice (n=5 mice/group). Data are shown as mean ± SEM.

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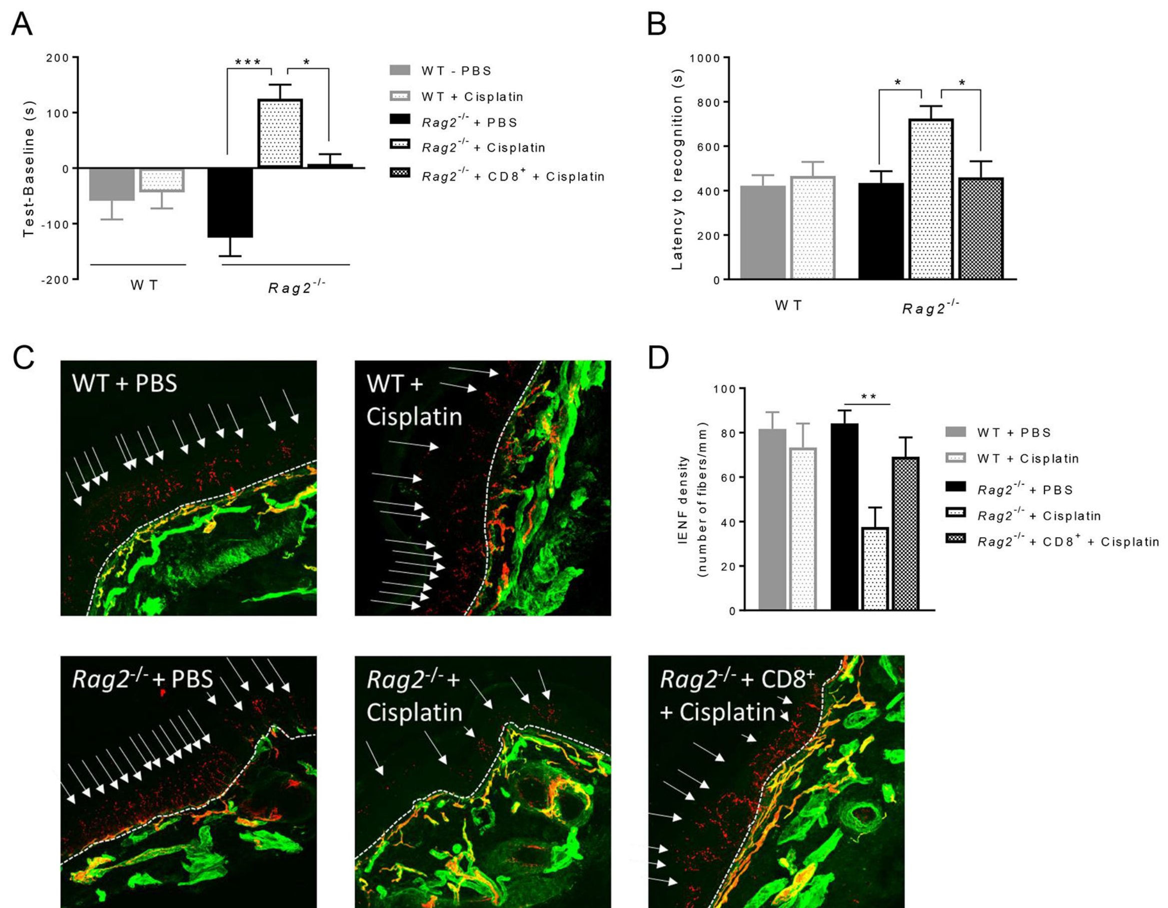

Figure 2. Effects of CD8^{+} T cells on spontaneous pain, numbness and intra-epidermal nerve fiber (IENF) density after cisplatin treatment

Female WT mice, *Rag2*$^{-/-}$ mice, and *Rag2*$^{-/-}$ mice reconstituted with CD8^{+} T cells were treated with cisplatin or PBS as in Figure 1. **(A)** Conditioned place preference (CPP) paradigm was performed 3 weeks after cisplatin or PBS injection (days 21 to 28). Y-axis indicates the change in time spent in the bright (analgesic-paired chamber) between baseline and test. One-way ANOVA followed by Bonferroni's multiple-comparisons test: $F(4,40)=10.9$, $P<0.0001$ (n=9 mice/group). **(B)** The adhesive recognition test (ART) was performed on day 26 after cisplatin or PBS injection. One-way ANOVA followed by Bonferroni's multiple-comparisons test: $F(4,22)=5.12$, $P=0.004$ (n=6 mice/group). **(C)** Representative images of IENFs (PGP9.5, red and collagen, green) in the glabrous skin of the plantar surface of the hind paws 3 weeks after cisplatin or PBS. Dashed lines indicate the basement membrane; arrows indicate IENFs. **(D)** Quantification of IENF density, expressed as the number of nerve fibers crossing the basement membrane/length of the basement membrane (mm). One-way ANOVA followed by Bonferroni's multiple-comparisons test: $F(4,25)=4.87$, $P=0.005$ (n=6 mice/group). Data are shown as mean ± SEM. $***P<0.001$, $**P<0.01$, $*P<0.05$.

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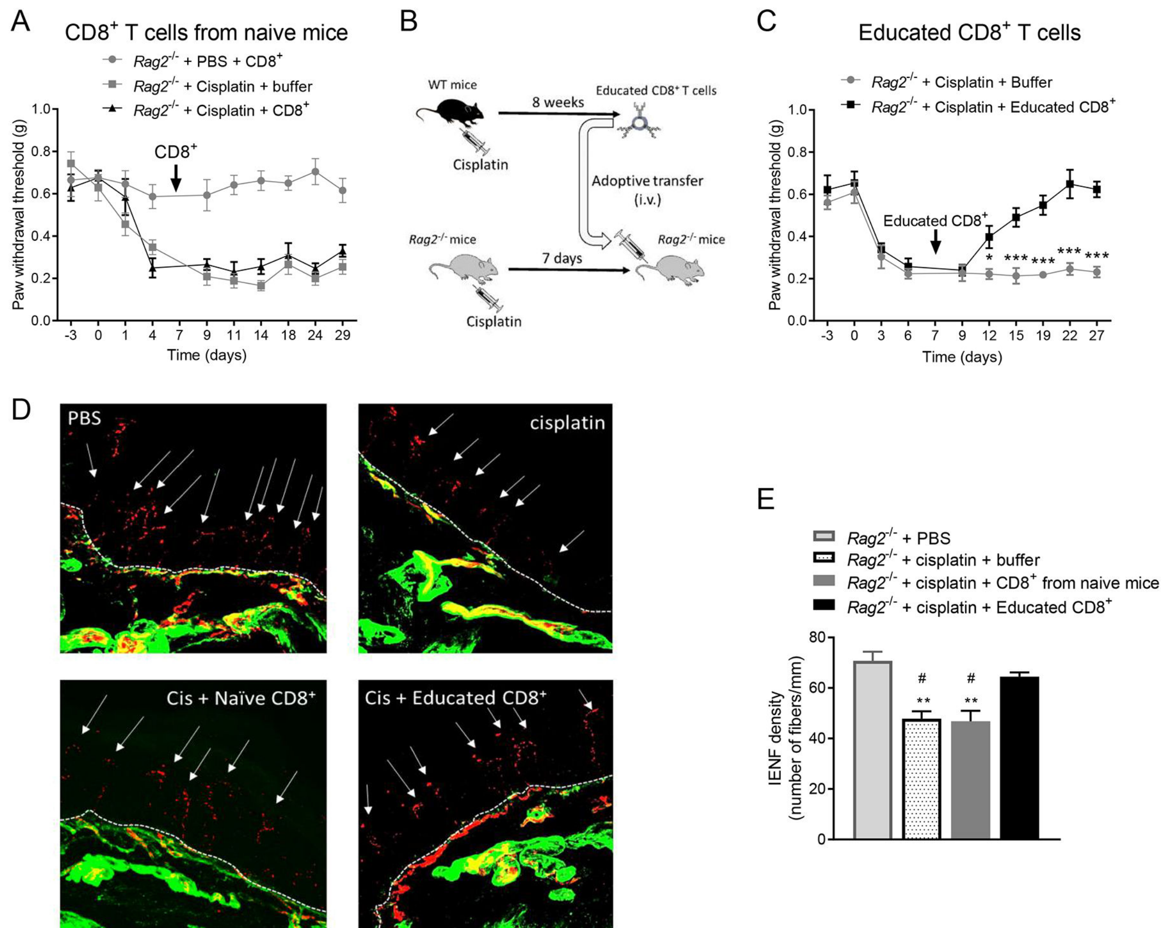

Figure 3. Effects of CD8+ T cells educated *in vivo* on established CIPN

(A) Time course of change in mechanical pain sensitivity. Female *Rag2*−/− mice were treated with cisplatin or PBS on days 0, 1, and 2. CD8+ T cells from naïve female WT mice were adoptively transferred on day 7 after start of cisplatin treatment (n=7 mice/group). **(B)** Schematic of *in vivo* education of CD8+ T cells. **(C)** Time course of mechanical pain sensitivity. *In vivo* educated CD8+ T cells obtained from WT female mice 8 weeks after cisplatin treatment were adoptively transferred on day 7 after start of cisplatin treatment into female *Rag2*−/− mice. Two-way repeated measures ANOVA interaction (time × CD8+ T cells): $F(9,108)=7.20$, $P<0.0001$ (n=7 mice/group). **(D)** Representative images of IENFs (PGP9.5, red and collagen, green) from *Rag2*−/− mice treated with PBS or cisplatin and reconstituted with CD8+ T cells from naïve mice or with educated CD8+ T cells. Dashed lines indicate the basement membrane; arrows indicate IENFs. **(E)** Quantification of IENF density, expressed as the number of nerve fibers crossing the basement membrane/length of the basement membrane (mm). One-way ANOVA followed by Bonferroni's multiple-comparisons test: $F(3,16)=13.8$, $P=0.001$ (n=5 mice/group). ** $P<0.01$, versus the PBS-treated group; # $p<0.05$ versus the cisplatin + educated CD8+ group. Data are shown as mean ± SEM. ***$P<0.001$, **$P<0.01$, *$P<0.05$.

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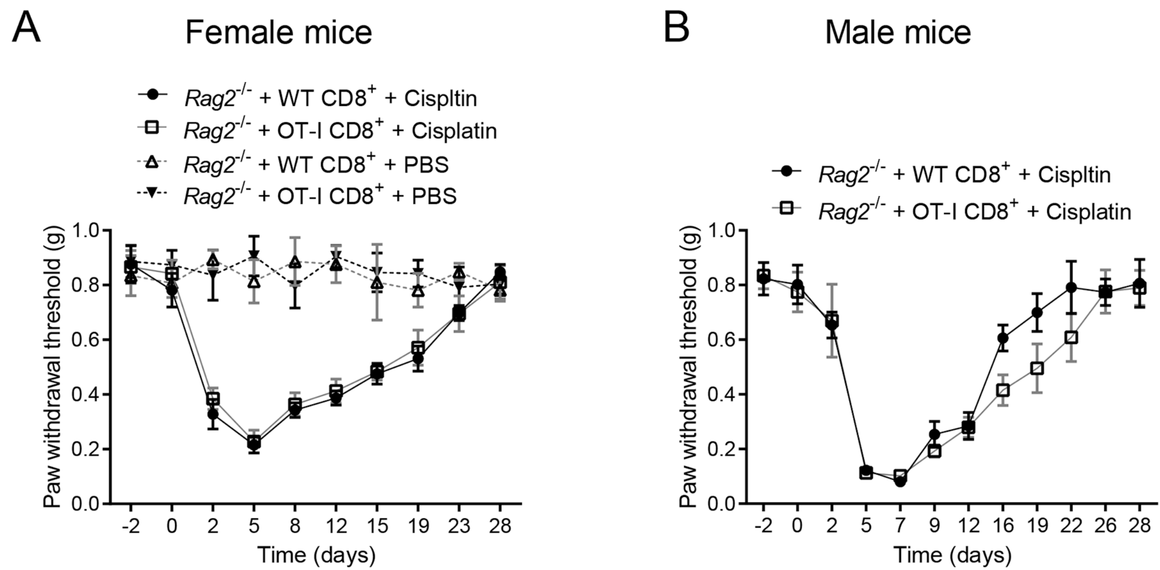

Figure 4. Contribution of TCR antigen recognition to resolution of chemotherapy-induced peripheral neuropathy

Female and male *Rag2*$^{-/-}$ mice were treated with cisplatin or PBS on days 0, 1, and 2. CD8^{+} T cells were adoptively transferred 10 days before start of cisplatin treatment. Mechanical pain sensitivity and resolution of allodynia. **(A)** Female *Rag2*$^{-/-}$ mice treated with PBS or cisplatin and reconstituted with CD8^{+} T cells from WT or OT-I mice (n=8 mice/group). **(B)** Male *Rag2*$^{-/-}$ mice treated with cisplatin and reconstituted with CD8^{+} T cells from WT or OT-I mice (n=6 mice/group). Data are shown as mean ± SEM.

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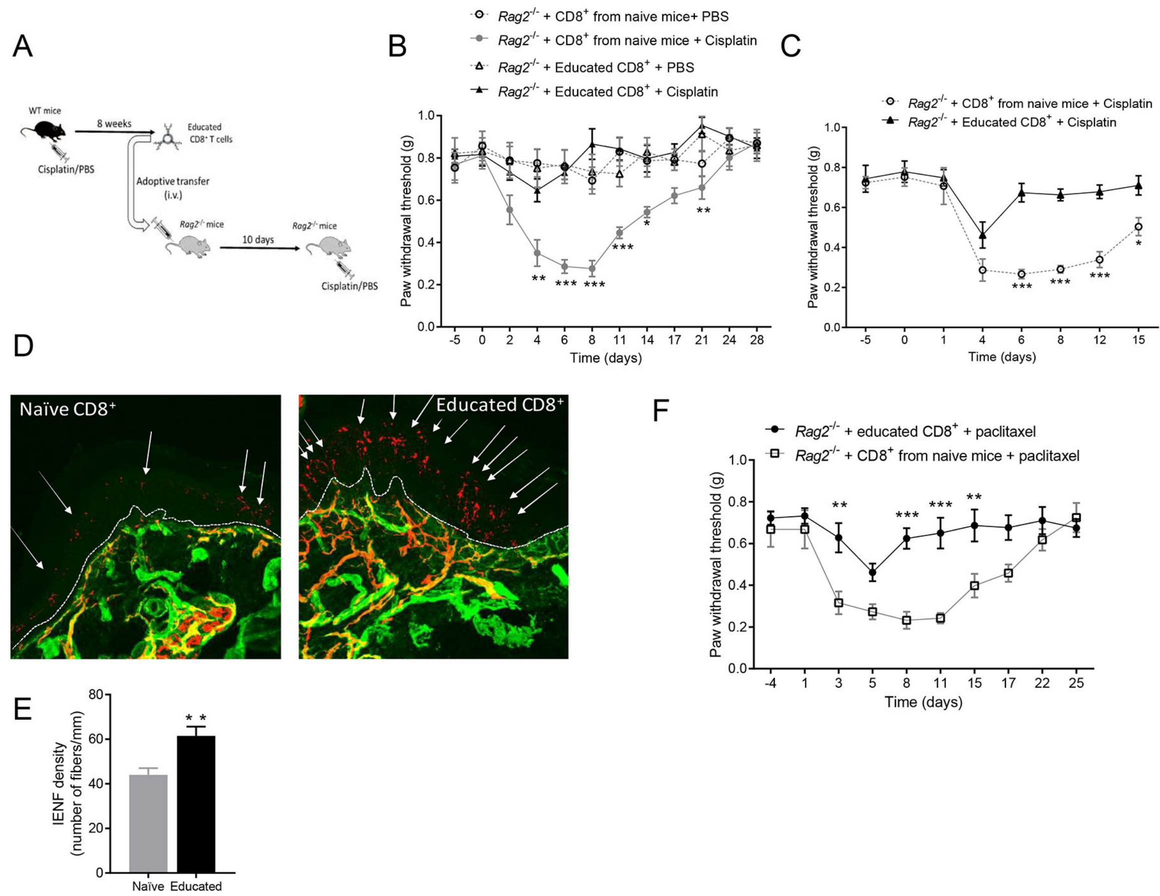

Figure 5. Effects of CD8+ T cells educated *in vivo* on chemotherapy-induced peripheral neuropathy

(A) Schematic of experiment to test the effect of reconstitution of *Rag2*$^{-/-}$ mice with CD8$^+$ T cells educated *in vivo* before cisplatin or PBS administration to the *Rag2*$^{-/-}$ mice. **(B)** Female *Rag2*$^{-/-}$ mice were treated with cisplatin or PBS on days 0, 1, and 2. CD8$^+$ T cells were adoptively transferred 10 days before start of cisplatin treatment. Mechanical pain sensitivity in female *Rag2*$^{-/-}$ reconstituted with educated CD8$^+$ T cells or CD8$^+$ T cells from naïve mice before cisplatin or PBS administration. Two-way repeated measures ANOVA interaction: $F(44,275)=5.05$, $P<0.0001$ (n=6 mice/group). **(C)** Mechanical allodynia in male *Rag2*$^{-/-}$ reconstituted with educated CD8$^+$ T cells or naïve CD8$^+$ T cells before cisplatin administration to the reconstituted *Rag2*$^{-/-}$ mice. Two-way repeated measures ANOVA interaction: $F(7,70) = 5.25$, $P<0.0001$ (n=6 mice/group). **(D)** Representative images of IENFs (PGP9.5, red and collagen, green) from cisplatin-treated female *Rag2*$^{-/-}$ mice previously reconstituted with educated CD8$^+$ T cells or CD8$^+$ T cells from naïve mice. Dashed lines indicate the basement membrane; arrows indicate IENFs. **(E)** Quantification of IENF density, expressed as the number of nerve fibers crossing the basement membrane/ length of the basement membrane (mm). T-test (t=3.42, *df*=8) (n=5 mice/group). **(F)** *Rag2*$^{-/-}$ mice were treated with paclitaxel on days 0 and 2. CD8$^+$ T cells from WT mice were adoptively transferred 10 days before start of paclitaxel treatment. Mechanical pain sensitivity I response to paclitaxel treatment in female *Rag2*$^{-/-}$ reconstituted with educated CD8$^+$ T cells or CD8$^+$ T cells from naïve mice. Two-way repeated measures ANOVA

interaction time × education: $F(9, 72) = 3.41$, $p=0.002$ (n=5mice/group). Data are shown as mean ± SEM. ***$P<0.001$, **$P<0.01$, *$P<0.05$.

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