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Involvement of $CD4^+$ and $CD8^+$ T-lymphocytes in the modulation of nociceptive processing evoked by CCL4 in mice

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ABSTRACT

Aims: To explore the mechanisms involved in the transformation of analgesia produced by low doses of CCL4 (pg/kg) to hyperalgesia when higher doses (ng/kg) are administered to mice.

Main methods: The unilateral hot plate test was used to assess thermal nociception. $CD3^+$, $CD4^+$ or $CD8^+$ blood cells were depleted with selective antibodies. Expression of CCR5 and IL-16 in lymphocytes was studied by flow cytometry and IL-16 blood levels were measured by ELISA. IL-16 and CD8 were detected by immunofluorescence.

Key findings: IL-16 and CCR5 expression were demonstrated in $CD4^+$ and $CD8^+$ T-lymphocytes by flow cytometry. Furthermore, CCL4-induced hyperalgesia was abolished by reducing circulating T-lymphocyte levels or by selectively depleting $CD4^+$ lymphocytes. In contrast, when the anti-CD4 antibody was acutely administered, CCL4 induced analgesia instead of hyperalgesia. A similar response was obtained when administering A-770041, that prevents CD4-mediated CCR5 desensitization by inhibiting $p56^{lck}$ kinase. As occurred with the analgesic effect evoked by low doses of CCL4, analgesia evoked by combining CCL4 and A-770041 was reverted by naloxone, naltrindole or an anti-met-enk antibody. Interestingly, flow cytometry assays showed that the number of $CD8^+$, but not $CD4^+$, T-cells expressing IL-16 is reduced after the acute administration of CCL4, a result compatible with the description that $CD8^+$-lymphocytes can rapidly release preformed IL-16. Accordingly, the rise in IL-16 blood concentration evoked by CCL4 was prevented after $CD8^+$ lymphocyte depletion.

Significance: CCL4-evoked hyperalgesia is related to the desensitization of CCR5 in $CD4^+$ T-cells and to the release of IL-16 from $CD8^+$ lymphocytes.

1. Introduction

CCL4 (MIP-1β) is a chemokine involved in the production of hypernociceptive responses associated to some experimental settings, such as neuropathy [1] or inflammation [2]. In accordance, its pronociceptive effects have been shown in mice after perineural [1] or intraplantar administration [3]. However, the modulation of nociception by CCL4 seems complex and it has recently been reported that the systemic administration of this chemokine to mice can evoke either hyperalgesic or analgesic responses on thermal nociception depending on the dose administered. In both circumstances, the effects produced by CCL4 are related to the release of molecules responsible for nociceptive modulation from circulating white blood cells, as demonstrated after leukocyte depletion treatments. Thus, the administration of very low doses of CCL4, in the order of pg/kg, leads to analgesic responses through the release of met-enk from $CD4^+$ lymphocytes [3] whereas higher ones, in the range of ng/kg, evoke thermal hyperalgesia by increasing the presence in plasma of different mediators such as CCL2, IL-1α, CXCL1, CXCL13 and IL-16 [4]. Thus, the administration of very low doses of CCL4, in the order of pg/kg, leads to opioid analgesia dependent on $CD4^+$ lymphocytes [3]. In this case, the detection of systemic analgesia in the presence of elevated blood levels of met-enk and its reversion by a selective δ-opioid receptor antagonist [3], strongly indicate that this effect is due to the activation of peripheral δ-opioid

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receptors, a mechanism already established by using selective agonists [4].

In contrast, the administration of higher doses of CCL4, in the range of ng/kg, evokes thermal hyperalgesia by increasing the presence in plasma of different mediators such as CCL2, IL-1α, CXCL1, CXCL13 and IL-16 [5]. In particular, a crucial role in this transformation from analgesia into hyperalgesia seems to be played by IL-16, a cytokine initially named Lymphocyte Chemoattractant Factor due to its outstanding ability to modulate lymphocyte functions [6]. The relevance of IL-16 in the hyperalgesic effect evoked by CCL4 is supported by the fact that the neutralization of IL-16 with an antibody provokes the complete inhibition of this hyperalgesic response and leads to its transformation into analgesia. In addition, the reversion of the hyperalgesia evoked by the administration of IL-16 when blocking the action of some hyperalgesic mediators involved, such as IL-1α, CXCL1 or CCL2 [5] reinforces the notion that IL-16 is an initial trigger that favors the release of these pronociceptive molecules. The high affinity of IL-16 for CD4 receptors [7,8] present in $CD4^+$ T-lymphocytes makes their participation in the hyperalgesic response induced by CCL4 likely. Supporting this view, CD4 activation can promote the release of chemokines or interleukins with hyperalgesic properties from $CD4^+$ lymphocytes [9,10] including the aforementioned CCL2 [11]. In addition, the activation of CD4 by IL-16 can provoke the inhibition of the transductional events triggered by CCR5 stimulation [12] and thus, could interfere with CCL4-mediated responses.

The experiments presented here were designed to explore the mechanisms involved in this switch from analgesia into hyperalgesia observed when doses of CCL4 are increased, considering that IL-16, the key molecule involved in CCL4-induced hyperalgesia, is an outstanding modulator of $CD4^+$-lymphocyte activity [9]. Since a previous study showed that CCL4-induced hyperalgesia can be prevented when white blood cells are depleted with cyclophosphamide [5], we initially have tried to elucidate the possible role played by $CD4^+$ and $CD8^+$ lymphocytes in CCL4-evoked hyperalgesia. We have further considered which of these cell lines of lymphocytes could be responsible for the release of IL-16 after the administration of a hyperalgesic dose of CCL4. Also, the possibility that IL-16-induced CD4 receptor activation [13,14] could participate in the inhibition of the analgesic mechanisms triggered by low doses of CCL4 acting through CCR5 was explored.

2. Materials and methods

2.1. Animals

Swiss male mice 6–8 week old from the Animalario of the Universidad de Oviedo (Reg. 33044 13A) exposed to a light-dark cycle of 12 h and with free access to water and food, were used. Experiments were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Spain) and performed according to the guidelines of European Communities Council Directive (2010/63/EU) for animal experiments.

2.2. Unilateral hot plate test

As described [5], mice were gently restrained and the plantar side of the tested paw was placed on a hot plate (IITC, Life Science, California, USA) set at 49 °C. Withdrawal latencies from the heated surface of each hind paw were measured separately at 2-min intervals and the mean was considered. A cut-off of 30 s was established. A basal measurement was performed in each paw in the morning of the experimental day (9:00–11:00 h; data not shown) to habituate mice to handling and the testing environment and experimental measures were taken between 14:00 and 19:00 h in an isolated room with controlled temperature and humidity. To prevent possible bias due to litter differences or daily variables, mice with different treatments or doses were tested each experimental day.

In depletion experiments with anti-CD3, anti-CD4 or anti-CD8 antibodies, nociceptive withdrawal latencies were always taken before the obtention of blood samples necessary to confirm depletion by white cell counting or flow cytometry analysis. Thus, although in these particular experiments the same mice were used for *in vivo* and *in vitro* assays, blood was always collected right after measuring withdrawal latencies in order to avoid any interference of blood extraction with nociceptive measurements.

2.3. Drugs

Subcutaneous (s.c.) administration was performed under the fur of the neck in a volume of 10 ml/kg. CCL4 (100 ng/kg; ProSpec) was s.c. administered 1 h before testing dissolved in distilled water with 0.1% bovine serum albumin (Sigma). The CCR5 antagonist DAPTA (5 mg/kg; Tocris), the opioid receptor antagonist naloxone (3 mg/kg; Tocris) and the selective δ-opioid receptor antagonist naltrindole (1 mg/kg; Tocris) were dissolved in saline and administered s.c. 1 h, 15 min and 30 min before testing, respectively. The $p56^{lck}$ inhibitor A-770041 (0.01–3 mg/kg; Axon) was solved in 3% DMSO/distilled water and administered s.c. 1 h before testing.

The anti-met-enk antibody (1 μg/kg; AB1975, Millipore) was solved in PBS (Phosphate Buffered Saline) and administered i.v. 1 h before testing in a volume of 5 ml/kg. The acute administration of the anti-CD4 antibody (10–100 ng/kg; Stemcell 60,029) was performed s.c. 1 h before testing.

When antibodies were used to produce lymphocyte depletion [15], anti-CD3 (1 μg, BioLegend 100,201), anti-CD4 (30 μg, Stemcell 60,029) or anti-CD8 (20 μg; SouthernBiotech 1550–01) antibodies were administered i.p., 24 h before testing in a volume of 300 μl of PBS. In all cases, control mice received an injection of the corresponding IgG isotype (eBioscience), rat IgG2aκ for the anti-CD8 antibody or rat IgG2bκ for anti-CD3 and anti-CD4 antibodies.

2.4. White cell count and flow cytometry analysis

2.4.1. White cell count

Blood obtained from the facial vein of mice anesthetized with isoflurane (3%, Isoflo®, Zoetis) was collected in an Eppendorf tube containing 10 μl of EDTA (0.5 M, pH = 8). The white cell population was quantified by a differential hematology analyzer (Abacus junior vet, Diatron) that, using a laser-based optical technique counts cells based on their impedance when passing through a small aperture. The apparatus gives separated counts of the total number of white blood cells as well as the number of lymphocytes, neutrophil granulocytes and mid-size cells including monocytes together with basophils and eosinophils.

2.4.2. Flow cytometry

As before, peripheral blood was collected from the facial vein in EDTA-coated tubes and samples were incubated with red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH = 7.4; Sigma) for 10 min at 4 °C and centrifuged at 250*g* for 5 min. The pellet was resuspended in 100 μl PBS containing a premix of antibodies and incubated for 20 min at room temperature (RT) in the dark. To assess CCR5 surface expression on T-cell subsets the anti-CCR5-APC (clone HM-CCR5, Biolegend; 10 μg/ml), the anti-CD3-FITC (clone 145-2C11, BioLegend; 10 μg/ml), the anti-CD4-PE (clone GK1.5, BioLegend; 4 μg/ml) and the anti-CD8-Alexa Fluor 700 (clone 53–6.7, BioLegend; 10 μg/ml) were used.

For intracellular detection of IL-16 on $CD4^+$ and $CD8^+$ cells, samples were initially stained as described above with the anti-CD3-FITC, the anti-CD4-APC and the anti-CD8-Alexa Fluor 700. Afterwards, samples were fixed and permeabilized by using BD Cytofix/Cytoperm solution kit (BD Biosciences) as previously described [16]. Briefly, samples were incubated with Cytofix/Cytoperm solution for 20 min at 4 °C and washed twice with 1× Perm/Wash buffer (BD Biosciences).

Subsequently, samples were stained with anti-IL-16-PE (clone 14.1, Biolegend; 1:100) for 30 min at 4 °C in the dark and washed once more with Perm/Wash buffer.

Samples were resuspended in PBS, acquired on a CytoFLEX flow cytometer (Beckman Coulter) and analyzed using CytExpert 2.3 software. Gating strategy was determined by staining the samples with all the antibodies listed above except anti-CCR5 or anti-IL-16 in the assays performed herein. The percentage of $CCR5^+$ and $IL\text{-}16^+$ cells was calculated in each T-cell subset based on the gating previously determined in these fluorescence minus one (FMO) samples.

2.5. IL-16 enzyme-linked immunosorbent assay (ELISA)

Mice were anesthetized with isoflurane (3%, Isoflo®, Zoetis) and blood was collected by intracardiac puncture, placed in Eppendorf tubes and kept 2 h at RT. Tubes were centrifuged (1000 *g*, 15 min, 4 °C) and the supernatant was harvested and stored at −80 °C until use. A commercial ELISA kit (R&D) was used to determine serum levels of IL-16. Following manufacturer recommendations, 50 μl serum were used and plates were read at 450 nm in a microplate reader (Synergy H1, BioTek). The procedure was done in duplicate with independent samples coming from different mice.

2.6. Immunofluorescence assays

Blood was obtained from the facial vein and red blood cell lysis was performed with lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH = 7.4; Sigma) for 10 min on ice and centrifuged (1200 rpm, 5 min, 4 °C). This procedure was repeated three times and, after the last centrifugation, the resultant pellet was suspended in 30 μl 0.01 M PBS (pH = 7.4) and 5 μl of this cell suspension were spread on gelatin-coated slides (Super-Frost Plus, Menzel-Glaser) with a yellow tip sized for 20–200 μl micropipettes. Slides were dried for 2 h at RT, fixed with Cytofyx/Cytoperm (BD Biosciences) for 20 min on ice and kept in the dark. From this moment, the buffer used was always x1 Perm/Wash buffer (BD Biosciences). After washing (2 × 10 min) with this buffer, slides were air dried for 1 h and stored at −20 °C.

On the experimental day, slides were initially maintained 1 h at RT, washed in buffer for 30 min (2 × 15 min) and immersed in buffer containing 5% goat serum for 30 min. Next, they were incubated in rat anti-CD8α antibody (Southern Biotech1550–01, 1:200) dissolved in 5% goat serum buffer at RT, washed (2 × 15 min) and further incubated with the secondary antibody Alexa Fluor 546 (Invitrogen A11081, 1:200) for 1 h. This later incubation and the subsequent steps were performed in complete darkness. After a 2 × 15 min wash, slices were exposed to the anti-IL-16 antibody (Abcam, 180,792, 1:500) in 5% goat serum and washed (2 × 15 min). Finally, slides were immersed in the secondary antibody Alexa Fluor 488 (Invitrogen A11034, 1:250) for 1 h, washed once again and mounted with DAPI Fluoromount-G™ (SouthernBiotech).

Immunostained samples were imaged using a BX61 Olympus microscope with a 20×/0.75 NA objective. Images were acquired with an Olympus DP-70 CCD camera using ultraviolet excitation (BP330–385), blue-excitation (BP470–490) and green-excitation (BP530–550) filters and the Olympus DP-controller 1.2.1.108 software and Olympus DP-manager 1.2.1.107. In double staining assays, the green color represents the fluorescence of the anti-IL-16 antibody and red corresponds to the fluorescence of anti-CD8 antibody. Merged images were obtained by using the ImageJ software. Some detailed images were taken by using a Leica TCS SP8 X confocal microscope (Leica Microsystems, Heidelberg GmbH) with a Leica DMI8 automatic fluorescence inverted microscope. A 488 nm white laser line was used to excite Alexa Fluor 488 (IL-16 staining), and a 556 nm white laser line to excite Alexa Fluor 546 goat anti-rat (CD8 staining). DAPI staining on nuclei was excited using a 405 nm blue diode laser. Images were acquired with a HC PL APO CS2 63×/1.40 oil and a zoom of 2.15. Merged images were obtained by using the Leica Application Suite X (LAS X) version 1.8.1 Copyright 1997–2015 Leica Microsystems CMS GmbH.

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism software, version 6. Mean values and their corresponding standard errors were calculated. When two variables were considered, comparisons among groups were performed by applying a previous two-way ANOVA followed by Bonferroni's correction to establish significance. This was the case for the depletion experiments with the CD3 antibody being treatment (antibody or corresponding IgG) and type of cell (white cells, lymphocytes, monocytes or granulocytes) the two variables, depletion experiments with CD4 and CD8 antibodies where treatment (CD4 or CD8 antibodies or their corresponding IgG isotypes) and type of cells ($CD4^+$ or $CD8^+$ lymphocytes) were the two variables considered, the measurement of paw latencies in mice receiving CD4 or CD8 antibodies and CCL4 being the two variables studied depletion treatment (CD4 or CD8 antibodies or the corresponding IgG) and the acute administration or not of s.c. CCL4 (basal or CCL4), the percentage of $CD4^+$ or $CD8^+$ lymphocytes that express IL-16 being the two variables treatment (saline or CCL4) and type of cell ($CD4^+$ or $CD8^+$ lymphocytes). In the resting experiments in which only one variable was considered, one-way ANOVA analysis was followed by the Tukey's test to compare the effects induced by several treatments. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Involvement of lymphocytes in CCL4-induced hyperalgesia

In accordance with a previous report [5], the s.c. administration of 100 ng/kg of CCL4 1 h before testing evoked a hyperalgesic response detected by the decrease in nociceptive latencies below the basal ones (Fig. 1A). Demonstrating the participation of CCR5, this response was prevented by the administration of the selective antagonist DAPTA (s.c.; 5 mg/kg; 30 min before testing) (Fig. 1A).

Since the involvement of white blood cells in CCL4-evoked hyperalgesia has been described [5], we have explored the possible role of lymphocytes. Treatment with an anti-CD3 antibody (1 μg, 24 h before) significantly reduced total circulating white blood cells about 35% (from 4.3×10^6 *vs* 2.8×10^6 cells/ml, Fig. 1C) that was, in fact, exclusively related to the absence of lymphocytes. Thus, granulocyte content was almost identical in control and antibody-treated groups (Fig. 1C) and mid-size cell concentration was also undistinguishable between groups (Fig. 1C). In contrast, the treatment with the anti-CD3 antibody led to a 46% reduction of lymphocyte number (3.42×10^6 *vs* 1.83×10^6 cells/ml, Fig. 1C). When the effect of a hyperalgesic dose of CCL4 (100 ng/kg) was assessed in behavioral assays in these lymphocyte-depleted mice, the hyperalgesic effect was completely abolished (Fig. 1B).

Considering the involvement of CCR5 in CCL4-evoked hyperalgesia, an initial approach was to elucidate which lymphocyte subset, $CD4^+$ or $CD8^+$, could express CCR5. With this aim, fluorescence-activated cell sorting assays were performed in blood samples. Among all cells marked with the anti-$CD3^+$ antibody, the percentage expressing CCR5 was 43.73 ± 6.86% (Fig. 2A). When these $CD3^+$ cells were sorted by using selective anti-CD4 and anti-CD8 antibodies, a similar CCR5 expression was detected in both $CD4^+$ (40.37 ± 6.92%) (Fig. 2B) and $CD8^+$ (47.95 ± 6.35%) T-cells (Fig. 2C), respectively.

In addition, since IL-16 is a crucial mediator of the hyperalgesia evoked by CCL4 [5], its presence in both subsets of lymphocytes was also checked. As before, this goal was achieved by performing flow cytometry analysis of blood samples with anti-$CD3^+$, anti-$CD4^+$, anti-$CD8^+$ and anti-IL-16 antibodies after permeating cells to allow the detection of intracellular IL-16. This interleukin was expressed in 90.3 ± 2.6% $CD3^+$ T-cells (Fig. 3A), corresponding to 87.7 ± 3.2% $CD4^+$ (Fig. 3B) and 98.6

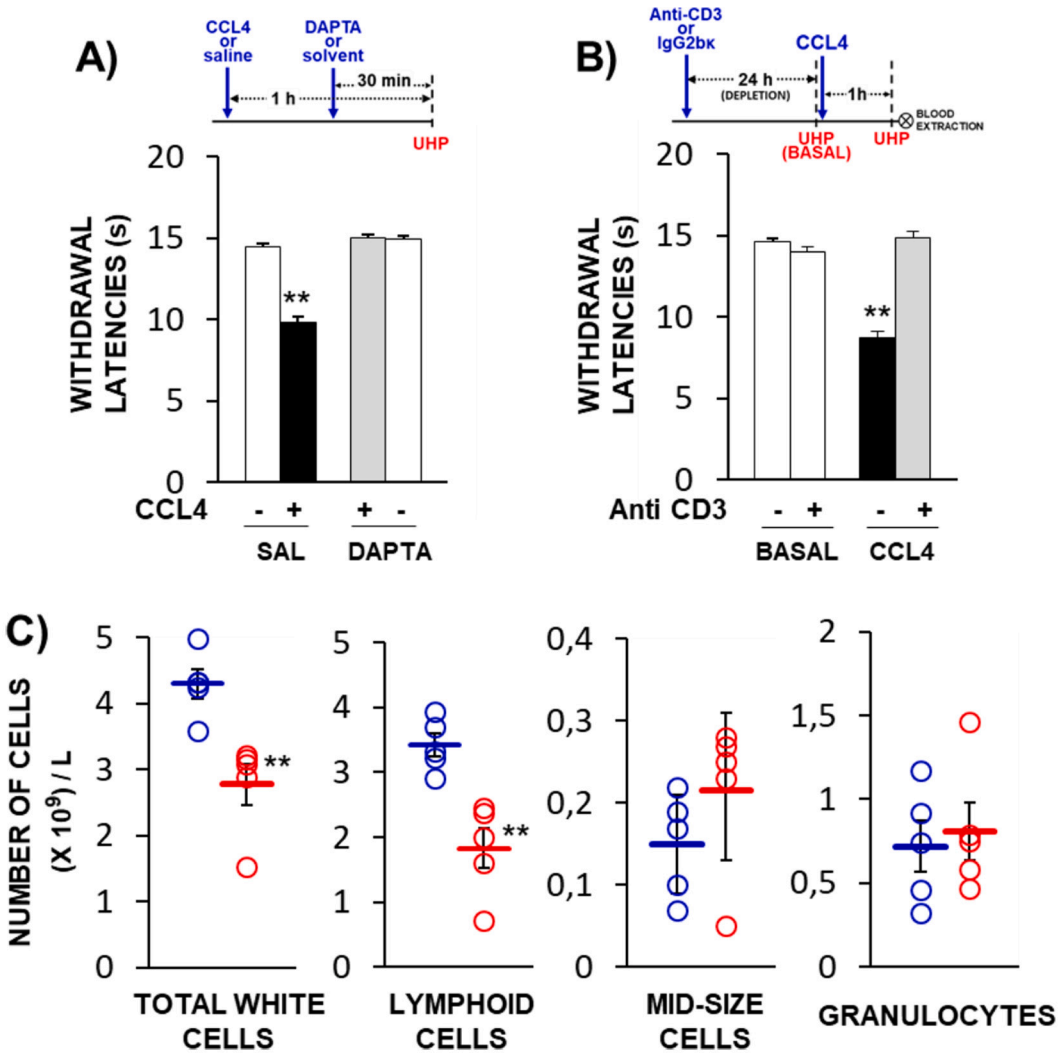

Fig. 1. A) Thermal hyperalgesia evoked by the administration of 100 ng/kg of CCL4 (s.c.; 1 h before testing) and its prevention by the CCR5 selective antagonist DAPTA (5 mg/kg, s.c., 1 h before testing). ($N = 5$–6). $^{**}P < 0.01$ compared with saline (SAL)-treated group, Tukey's test. On top, a diagram of the experimental design is shown.

B) Mice received the i.p. administration of anti-CD3 antibody (1 μg/mouse; N = 5) or the corresponding IgG2bκ isotype (1 μg/mouse; N = 5) and 24 h later, withdrawal latencies were taken before any further treatment (BASAL). Next, mice received the acute administration of CCL4 (100 ng/kg) and 1 h later withdrawal latencies were measured again ($n = 5$). $^{**}P < 0.01$ compared with the corresponding basal latencies, Bonferroni's correction. On top, a diagram of the experimental design is shown.

C) Effect of i.p. treatment 24 h before with either anti-CD3 antibody (1 μg/mouse; N = 5) or IgG2bκ isotype (1 μg/mouse; N = 5) on the total number of circulating white blood cells, lymphocytes, mid-size cells and granulocytes. Individual data are represented as red (anti-CD3 antibody) or blue (isotype) circles and their mean and corresponding S.E. appears as a short red or blue line, respectively. $^{**}P < 0.01$ compared with isotype-treated group, Bonferroni's correction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

± 0.8% $CD8^+$ cells (Fig. 3C).

Bearing in mind that the expression of CCR5 and IL-16 in a large population of either $CD4^+$ and $CD8^+$ T-lymphocytes makes the involvement of both cell types in CCL4-mediated hyperalgesia feasible, we performed behavioral experiments in mice after the selective depletion of $CD4^+$ or $CD8^+$ blood T-lymphocytes. Initially, the number of circulating $CD4^+$ cells assessed by flow cytometry assays was significantly reduced after the *in vivo* treatment with the anti-CD4 antibody (30 μg, 24 h before), as compared with non-depleted mice receiving the corresponding isotype. Thus, the $CD4^+$ population was 74 ± 1.1% of $CD3^+$ lymphocytes in isotype-treated mice, but only 9 ± 1.6% in mice receiving the anti-CD4 antibody (Fig. 4A-B).

Interestingly, the hyperalgesic response to the administration of 100 ng/kg of CCL4 was completely absent in these mice in which the majority of $CD4^+$-lymphocytes were eliminated (Fig. 5A). On the other hand, mice receiving the anti-CD8 antibody (20 μg, 24 h before) showed a remarkable reduction of the number of circulating $CD8^+$ lymphocytes. Although this population was already a minority in isotype-treated mice, constituting 24.6 ± 1.6%, the percentage decreased up to 9.6 ± 1.7% after treatment with the anti-CD8 antibody (Fig. 4C-D). The *in vivo* response observed after the administration of 100 ng/kg of CCL4 to these $CD8^+$ T-cell-depleted mice was dramatically different to the one observed in mice treated with the corresponding IgG2aκ isotype, since the reduction of $CD8^+$ lymphocytes not only inhibited the hyperalgesic response evoked by 100 ng/kg of CCL4 but transformed it to a remarkable analgesic effect (Fig. 5B). Moreover, this effect was prevented by the administration of naloxone (3 mg/kg, s.c., 15 min before testing), naltrindole (1 mg/kg, s.c., 30 min before testing), or the administration of an anti-met-enk antibody (1 μg/kg, i.v., 60 min before testing) (Fig. 5C), thus supporting the notion that it is qualitatively similar to that evoked by doses of CCL4 in the range of pg/kg [3].

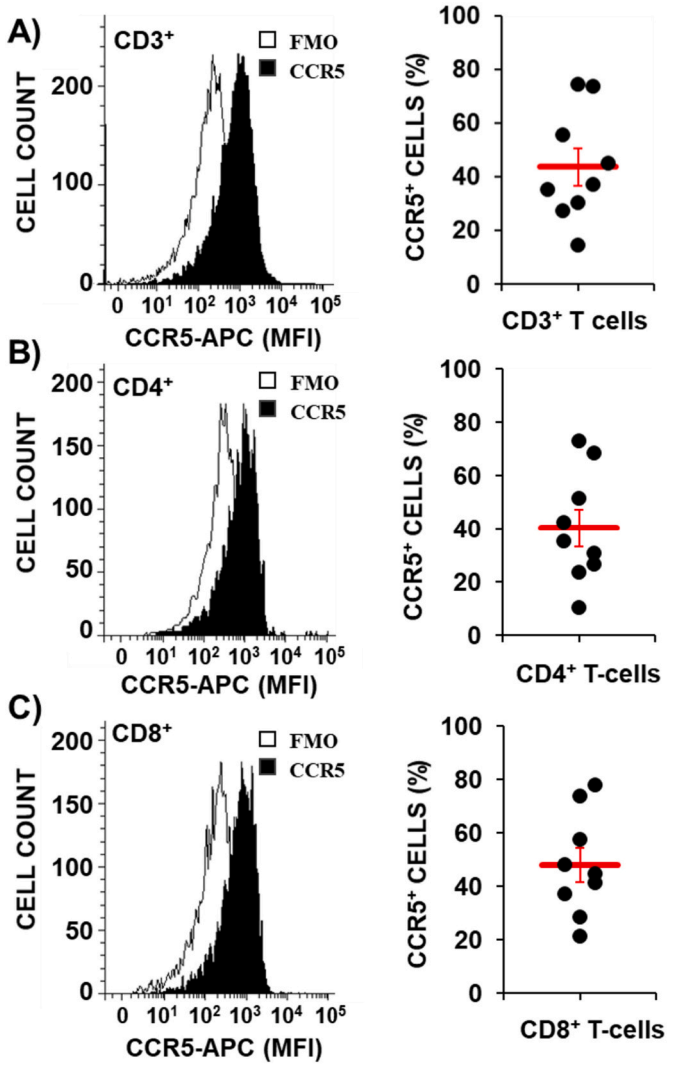

Fig. 2. Determination by flow cytometry of CCR5 surface expression on $CD3^+$, $CD4^+$ and $CD8^+$ T-cell subsets coming from peripheral blood collected from naïve mice. Histograms of the left-hand side depict representative examples of the fluorescence distribution (mean fluorescence intensity, MFI) detected in the APC channel (CCR5-APC) in cells coming from one sample showing the fluorescence for the FMO sample (without CCR5 staining) and its CCR5-stained counterpart obtained in $CD3^+$ (A), $CD4^+$ (B) and $CD8^+$ (C) cells.
The graphs of the right-hand side show percentages of cells expressing CCR5 on $CD3^+$ (A), $CD4^+$ (B) and $CD8^+$ (C). Dots correspond to individual values obtained in each sample and red lines represent the calculated mean and the corresponding S.E. ($n = 9$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

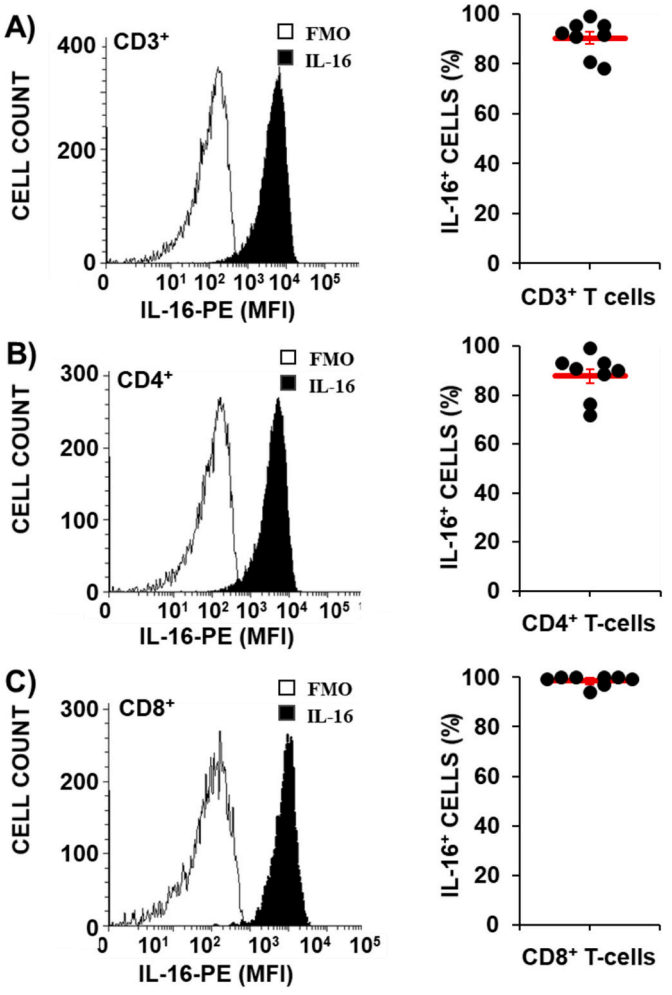

Fig. 3. Determination by flow cytometry of intracellular IL-16 expression on $CD3^+$, $CD4^+$ and $CD8^+$ T-cell subsets coming from peripheral blood collected from naïve mice. The histograms of the left-hand side depict representative examples of the fluorescence distribution (mean fluorescence intensity, MFI) detected in the PE channel (IL-16-PE) in cells coming from one sample showing the fluorescence for the FMO sample (without IL-16 staining) and its IL-16-stained counterpart obtained in $CD3^+$ (A), $CD4^+$ (B) and $CD8^+$ (C) cells.
The graphs of the right-hand side show percentages of cells expressing IL-16 on $CD3^+$ (A), $CD4^+$ (B) and $CD8^+$ (C). Dots correspond to individual values obtained in each sample and red lines represent the calculated mean and the corresponding S.E. ($n = 8$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Involvement of CD4 receptors in the hyperalgesia evoked by CCL4 administration

Since the CD4 receptor is the main target for IL-16, we have studied whether its acute blockade could prevent the establishment of hyperalgesia after CCL4 administration. The reduction of withdrawal latencies evoked by 100 ng/kg of CCL4 was dose-dependently inhibited after the acute administration of an anti-CD4 antibody (10–1000 ng/kg, s.c., 1 h before) and transformed into analgesia after the administration of the maximal dose of the antibody (Fig. 6B). Since this result supported the participation of CD4 receptors in CCL4-evoked hyperalgesia and the binding of IL-16 to CD4 receptors rapidly evokes the desensitization of CCR5 receptors [12], we assayed the effect of A-770041, a drug able to inhibit $p56^{lck}$, the kinase responsible for CCR5 desensitization triggered by CD4. The hyperalgesic effect evoked by CCL4 was dose-dependently prevented by the administration of A-770041 (0.01–3 mg/kg, s.c., 1 h before), being progressively transformed to analgesia when the maximal dose was injected (Fig. 6C).

In order to determine if this analgesic effect is mediated by opioid mechanisms, we assayed the effect of different drugs able to block opioid mechanisms. Interestingly, analgesia obtained when combining the administration of a hyperalgesic dose of CCL4 with 3 mg/kg of A-770041 was prevented after the administration of the non-selective opioid receptor antagonist naloxone (3 mg/kg, s.c., 15 min before), the selective δ-opioid receptor antagonist naltrindole (1 mg/kg, s.c., 30 min before) or an anti-met-enk antibody (1 μg/kg, i.v., 1 h before) (Fig. 6D). The administration of either A-770041, naloxone, naltrindole or the anti-met-enk antibody at the maximal doses assayed did not modify withdrawal latencies when administered alone.

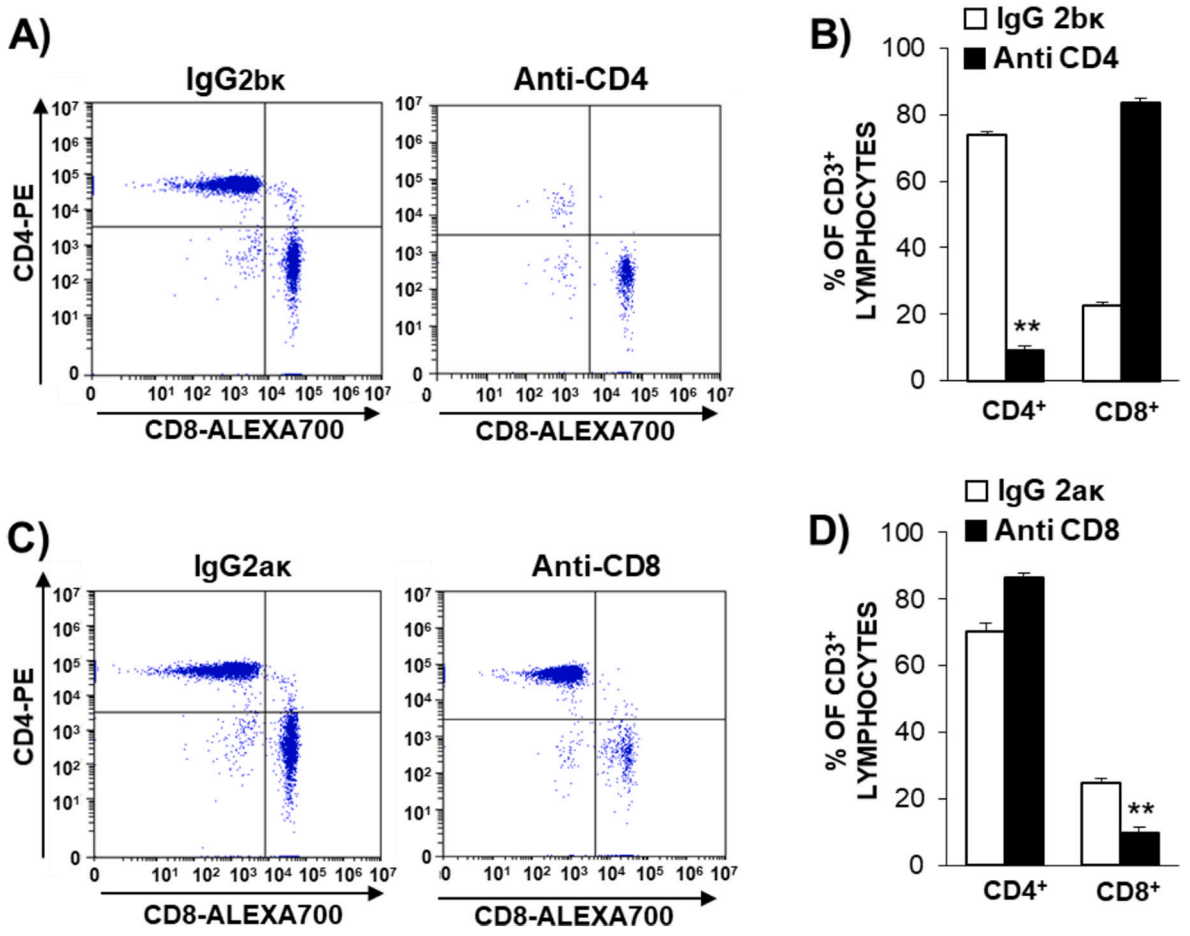

Fig. 4. A-B) Flow cytometry analysis of peripheral blood $CD3^+$ cells from mice i.p. treated 24 h before with either an anti-CD4 antibody (30 μg/mouse) or its corresponding isotype, IgG2bκ. Representative flow cytometry profiles of each treatment are shown in A, and the mean percentages of $CD4^+$ and $CD8^+$ cells detected in mice receiving IgG2bκ ($n = 5$) or anti-$CD4^+$ antibody (n = 5) are represented in B. C—D) Flow cytometry analysis of peripheral blood $CD3^+$ cells from mice i.p. treated 24 h before with either an anti-CD8 antibody (20 μg/mouse) or its corresponding isotype, IgG2aκ. Representative flow cytometry profiles of each treatment are shown in C, and the mean percentages of $CD4^+$ and $CD8^+$ cells detected in mice receiving IgG2aκ ($n = 5$) or anti-$CD8^+$ antibody (n = 5) are represented in D. $^{**}P < 0.01$ comparing with mice treated with the corresponding IgG isotype, Bonferroni's correction.

3.3. Role of $CD8^+$ T-lymphocytes in the switch from analgesia to hyperalgesia when doses of CCL4 are increased

The expression of CCR5 and IL-16 in a wide percentage of $CD4^+$ and $CD8^+$ lymphocytes and the modification of CCL4-induced hyperalgesia after the selective depletion of each lymphocyte subset suggests that these cells could act as a relevant source of IL-16 after CCL4 administration. To check this possibility, we assessed by flow cytometry whether the number of $CD4^+$ and $CD8^+$ T-cells expressing IL-16 could be altered after the acute administration of 100 ng/kg of CCL4. As shown in Fig. 7, the number of $CD4^+$ T-cells expressing IL-16 was very similar in mice treated with saline (86.6 ± 2.7%) or with CCL4 (85.5 ± 4%, $P = 0.8088$) (Fig. 7A-B). By contrast, 97.5 ± 0.9% $CD8^+$ T-cells of mice treated with saline express IL-16, but this percentage was significantly reduced in mice receiving the administration of 100 ng/kg of CCL4 (76.8 ± 3.4%, $P < 0.0001$) (Fig. 7C-D), thus suggesting that this chemokine is triggering IL-16 secretion from $CD8^+$ T-cells.

Furthermore, we have performed ELISA assays trying to confirm that the increase in IL-16 blood levels evoked by CCL4 could be related to the presence of circulating $CD8^+$ lymphocytes. As shown in Fig. 7E, the administration of 100 ng/kg of CCL4 evoked a significant augmentation of IL-16 blood concentration from 0.81±0.24 pg/ml measured in control mice treated with isotype and solvent up to 1.74 ± 0.37 pg/ml 1 h after receiving CCL4. However, this increase in blood IL-16 levels in response to CCL4 administration was completely prevented in mice pretreated with 20 μg of anti-CD8 antibody 24 h before to evoke the depletion of $CD8^+$ lymphocytes.

In accordance with the role played by $CD8^+$-lymphocytes in IL-16 release, double staining immunofluorescence assays with antibodies addressed against both targets confirmed the presence of IL-16 in $CD8^+$ cells. When quantifying the number of white blood cells expressing these antigens present in a single blood smear, we estimated that, from 37 cells identified by DAPI staining, 20 (54%) expressed IL-16 and 6 (16%) CD8. All these $CD8^+$ cells showed double staining CD8/IL-16. In Fig. 8A an example with 10 blood cells marked with DAPI is shown in which 6 of them are IL-16^+ and one shows double staining CD8/IL-16. As observed with greater magnification in a confocal microscope image, green fluorescence corresponding to IL-16 can be observed at the cytoplasmatic level, whereas red staining produced by the anti-CD8 antibody, is restricted to the plasmatic membrane (Fig. 8B).

4. Discussion

The experiments presented here were addressed to explore the processes responsible for the transformation of the analgesia induced by low systemic doses of CCL4 to a hyperalgesic effect mediated by IL-16 when the doses of this chemokine are increased from pg/kg up to ng/kg. Confirming the previous finding that white blood cells are required for CCL4-induced hyperalgesia [5], we describe here the involvement of circulating lymphocytes and try to characterize the role played by $CD4^+$

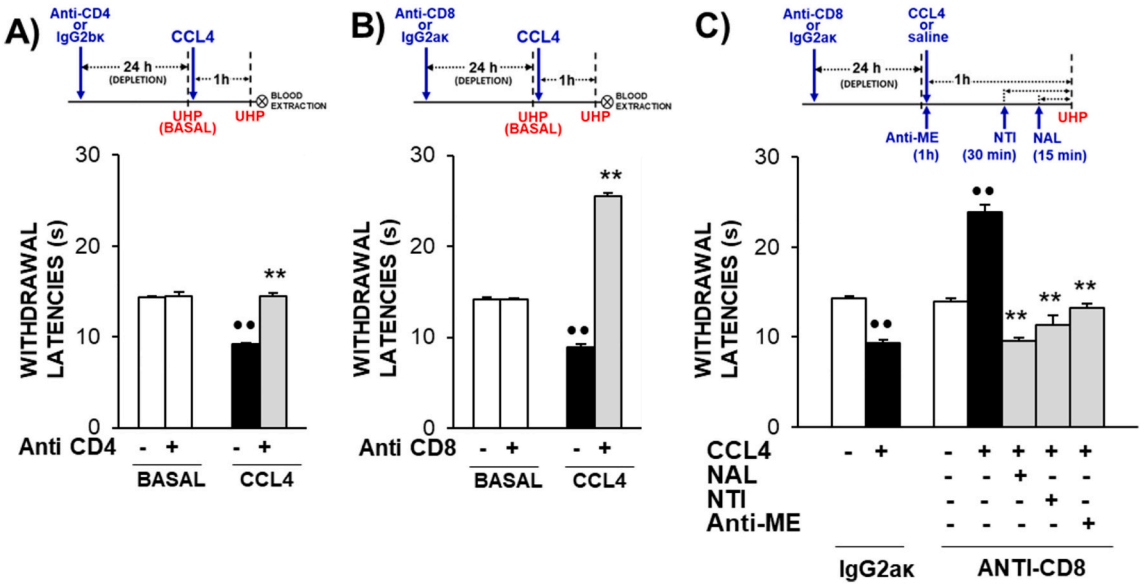

Fig. 5. Effects on the hyperalgesia evoked by CCL4 following the depletion of $CD4^+$ or $CD8^+$ T- lymphocytes measured in those mice whose blood flow cytometry analysis is shown in Fig. 4. A diagram of the experimental design is shown on top of each graph. A) Mice received the i.p. administration of 30 μg of an anti-CD4 antibody or the corresponding IgG2bκ isotype and 24 h later, withdrawal latencies were taken before any further treatment (BASAL). Next, mice received the acute administration of CCL4 (100 ng/kg) and 1 h later withdrawal latencies were measured again (n = 5). B) Mice received the i.p. administration of 20 μg of an anti-CD8 antibody or the corresponding IgG2aκ isotype and 24 h later, withdrawal latencies were taken before any further treatment (BASAL). Next, mice received the acute administration of CCL4 (100 ng/kg) and 1 h later withdrawal latencies were measured again ($n = 5$). Means and the corresponding standard error are represented •• $P < 0.01$ compared with the corresponding basal latencies, $^{**}P < 0.01$ compared with group receiving the isotype, Bonferroni's correction.
C) The analgesic effect measured in $CD8^+$-depleted mice in response to the administration of a normally hyperalgesic dose of CCL4 (100 ng/kg; s.c.; 1 h before testing) was prevented by the administration of the opioid receptor antagonist naloxone (NAL, 3 mg/kg, s.c., 15 min before), the selective δ-opioid receptor antagonist naltrindole (NTI, 1 mg/kg, s.c., 30 min before) or an anti-met-enk antibody (Anti-ME, 1 μg/kg, i.v., 1 h before). Means and corresponding standard errors are represented ($N = 5$). •• $P < 0.01$ compared with saline-treated, $^{**}P < 0.01$ compared with group treated with CCL4, Tukey's test.

and $CD8^+$ T-cell subsets. The participation of T-lymphocytes in a response driven by IL-16 could be considered a rather expected possibility, since this molecule can be released from lymphocytes or act on these cells exerting chemotactic and stimulating responses [9,17]. These effects are mainly due to the high affinity of IL-16 for CD4 receptors present in $CD4^+$ T-lymphocytes [13,14,18]. The possible involvement of this interleukin in nociceptive modulation has not yet been well characterized, although some clinical reports describe the increased presence of this cytokine in particular inflammatory settings. For instance, systemic sclerosis is a chronic disease in which trophic alterations of the skin are usually accompanied by severe itch and it has been proposed that its progression could run parallel to an increase of serum levels of IL-16 [19]. Moreover, augmented IL-16 blood concentration has been detected in patients with rheumatoid arthritis and a correlation of IL-16 levels with the expression of other inflammatory cytokines has been reported [20]. Its possible pathophysiological role is, however, discussed and, although some reports proposed that IL-16 could act as a relevant $CD4^+$ lymphocyte chemoattractant [21], other results suggest that the increased IL-16 levels does not accurately correlate with clinical signs [22]. As discussed below, our laboratory data could give further support to a hyperalgesic role of IL-16, that could activate CD4 receptors present in $CD4^+$ T-cells, leading to the rapid desensitization of CCR5 expressed in these cells, thus stopping the analgesic mechanisms triggered by CCR5 and opening the possibility for the establishment of a hyperalgesic state.

Our initial attempt was to elucidate whether CCL4-evoked hyperalgesia is related to circulating lymphocytes. The relationship between the hyperalgesic response activated by CCL4 and circulating white blood cells has been previously demonstrated [5], but the possible role of lymphocytes in this process remained to be elucidated. In our experiments, the observation that hyperalgesia following CCL4 administration is absent when the number of circulating lymphocytes is significantly reduced due to the administration of an anti-CD3 antibody 1 day before, demonstrates that the hyperalgesic effect induced by CCL4 requires the participation of these blood cells. In accordance with previous data [23,24,25], CCR5 was detected in both $CD4^+$ and $CD8^+$ T-cells. Taking into account the key role played by IL-16 and T-lymphocytes in CCL4-induced hyperalgesia and following a similar strategy, we checked whether this interleukin was present in the major lymphocyte subsets, $CD4^+$ and $CD8^+$. As previously described in human or murine T-cells [26,27,28], intracellular IL-16 was detected in a large percentage of $CD4^+$ and $CD8^+$ lymphocytes.

Flow cytometry experiments demonstrated that the administration of a high dose of antibodies against $CD4^+$ and $CD8^+$ T-lymphocytes 24 h before testing evokes the corresponding selective depletion. When mice with a drastic reduction in the percentage of $CD4^+$ lymphocytes received 100 ng/kg of CCL4, thermal hyperalgesia was completely absent, as occurred in mice whose $CD3^+$ T-cells were depleted by administering an anti-CD3 antibody. These results strongly suggest that $CD4^+$ lymphocytes, besides being crucial for the analgesia evoked by doses of CCL4 in the range of pg/kg [3], also play an outstanding role in the hyperalgesic response triggered by higher doses. Furthermore, the participation of $CD4^+$ lymphocytes in hyperalgesia seems related to the activation of CD4 receptors, as deduced from the inhibition observed when these receptors are acutely blocked by administering a low dose of the anti-CD4 antibody 1 h before testing. Since the dose administered (1 μg/kg) was about 1000 times lower than that used to deplete $CD4^+$ lymphocytes (20 μg/mouse) and its effect was established 1 h after administration, this inhibition seems unrelated to a possible lymphocyte depletion. In fact, the participation of CD4 receptors can be considered as a logical consequence of IL-16 involvement, since they are the main target where IL-16 binds to exert its functions [13,14,17]. Furthermore, as commented below, the acute administration of this anti-CD4 antibody not only inhibited hyperalgesia but even led to the induction of a remarkable analgesic effect.

The involvement of $CD4^+$ T-lymphocytes in both analgesia and

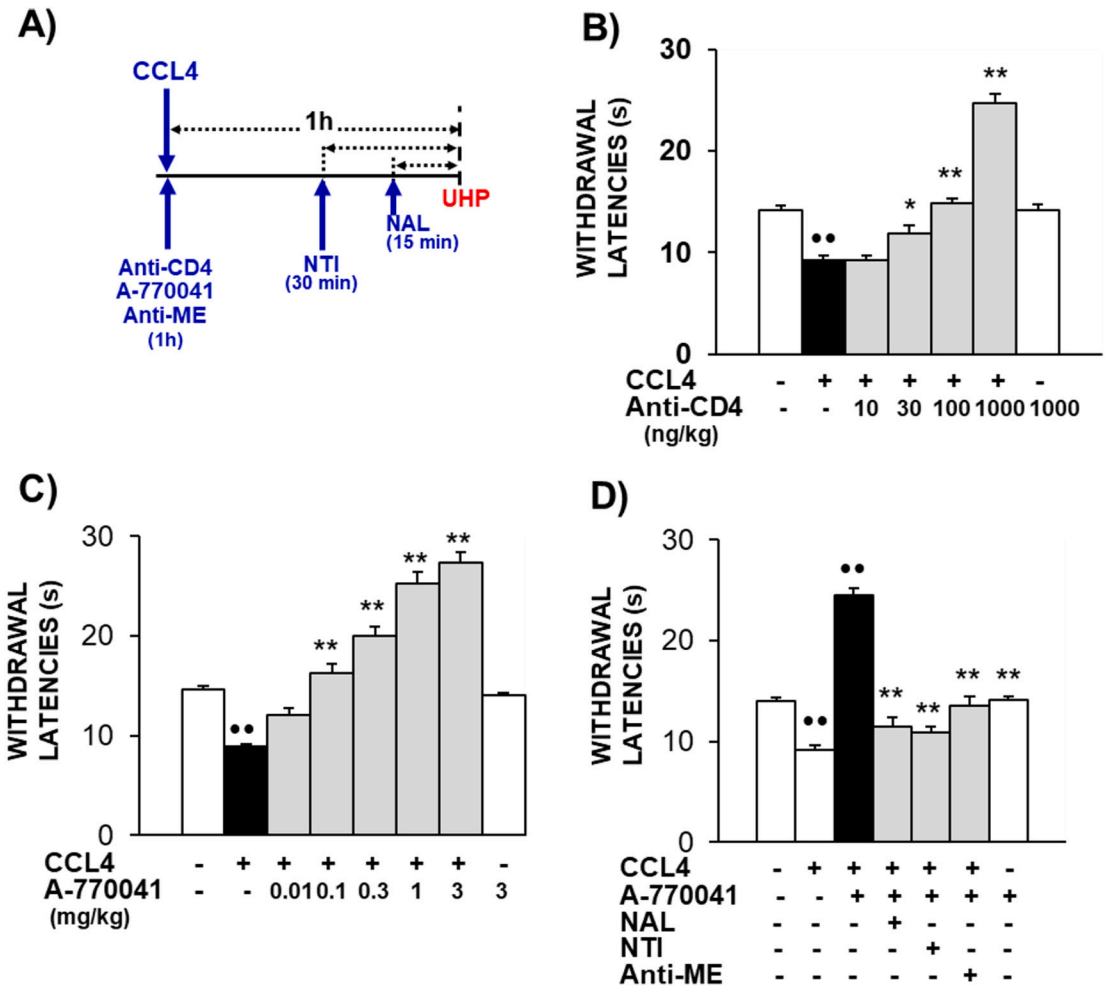

Fig. 6. A) Diagram of the general experimental design for B, C and D showing the time at which drugs were administered. B) Dose-dependent inhibition of the hyperalgesia evoked by CCL4 (100 ng/kg; s.c.; 1 h before) and further establishment of analgesia produced by the acute administration of an anti-CD4 antibody (10–1000 ng/kg; s.c.; 1 h before testing) ($n = 5$). C) Dose-dependent inhibition of the hyperalgesia evoked by CCL4 (100 ng/kg; s.c.; 1 h before) and further establishment of analgesia produced by the acute administration of the $p56^{lck}$ inhibitor A-770041 (0.01–3 mg/kg; s.c.; 1 h before testing) (n = 5). $^{**}P < 0.01$ compared with solvent-treated group, ••P < 0.01 compared with CCL4-treated group, Tukey's test. D) The analgesic effect measured in mice treated with 100 ng/kg of CCL4 and 3 mg/kg of A-770041 was prevented by the administration of the opioid receptor naloxone (NAL, 3 mg/kg, s.c., 15 min before), the δ-opioid receptor antagonist naltrindole (NTI, 1 mg/kg, s.c., 30 min before) or an anti-met-enk antibody (Anti-ME, 1 μg/kg, i.v., 60 min before). Means and corresponding standard errors are represented (n = 5–9). $^{**}P < 0.01$ compared with solvent-treated group, ••$P < 0.01$ compared with the group receiving CCL4 + A-770041, Tukey's test.

hyperalgesia evoked by CCL4 suggests that these cells might adopt opposite nociception-related roles in response to different doses of this chemokine. To understand this possible dual role, two hypothetical premises should be envisaged. Initially, the release of met-enk from $CD4^+$ T-lymphocytes that leads to analgesia after the administration of low doses of CCL4 [3] should be discontinued when higher doses are administered. In addition, other molecules responsible for the hyperalgesic reaction, as previously shown for IL-16, CCL2, IL-1α or CXCL1, should be released [5]. CCR5 unresponsiveness following CD4 receptor activation is recognized as a mechanism responsible for the inhibition evoked by IL-16 on CCL4-induced lymphocyte migration, a process dependent on the association of $p56^{lck}$ to the cytoplasmatic tail of CD4 [11,29,30]. This process could help to explain our results, since IL-16 could render CCL4 ineffective for the activation of CCR5 specifically in those cell lines expressing CD4, as typically $CD4^+$ lymphocytes are. In contrast, CCR5 responses could remain unaltered in those cells devoid of CD4 receptors. This interpretation can make the abovementioned result understandable showing the ability of CCL4 to evoke analgesia at high doses when CD4 receptors are neutralized by the acute administration of an anti-CD4 antibody. In accordance, the administration of a dose of the $p56^{lck}$ inhibitor A-770041 [31] able to avoid CD4 receptor-induced CCR5 desensitization *in vivo* [32] again produced the conversion of hyperalgesia in analgesia, suggesting that the disappearance of opioid analgesia evoked when doses of CCL4 are increased can be related to CCR5 unresponsiveness. Moreover, the analgesic effect obtained when combining CCL4 with A-770041 is abolished by blocking δ-opioid receptors or by neutralizing met-enk with an antibody, as occurred with the analgesia evoked by a low dose of CCL4 [3]. Both results demonstrate that CCL4 can maintain the ability of activating analgesic mechanisms in $CD4^+$ T-lymphocytes even in response to high doses of CCL4 when CCR5 desensitization is prevented.

Besides the participation of $CD4^+$-lymphocytes, we have also assessed the possible role played by $CD8^+$ cells. Flow cytometry experiments performed in mice treated with 20 μg of an anti-CD8 antibody one day before showed the selective depletion of $CD8^+$ lymphocytes. Interestingly, not only the hyperalgesic effect evoked by CCL4 was absent in these mice, as occurred in CD4-depleted mice, but, in addition, a remarkable analgesic effect was observed after the administration of 100 ng/kg of CCL4. Again, this analgesic response was blocked by either naloxone, naltrindole or an anti-met-enk antibody, strongly suggesting the participation of a similar analgesic mechanism to that operating when low doses of CCL4, in the pg/kg order, are administered [3]. Since

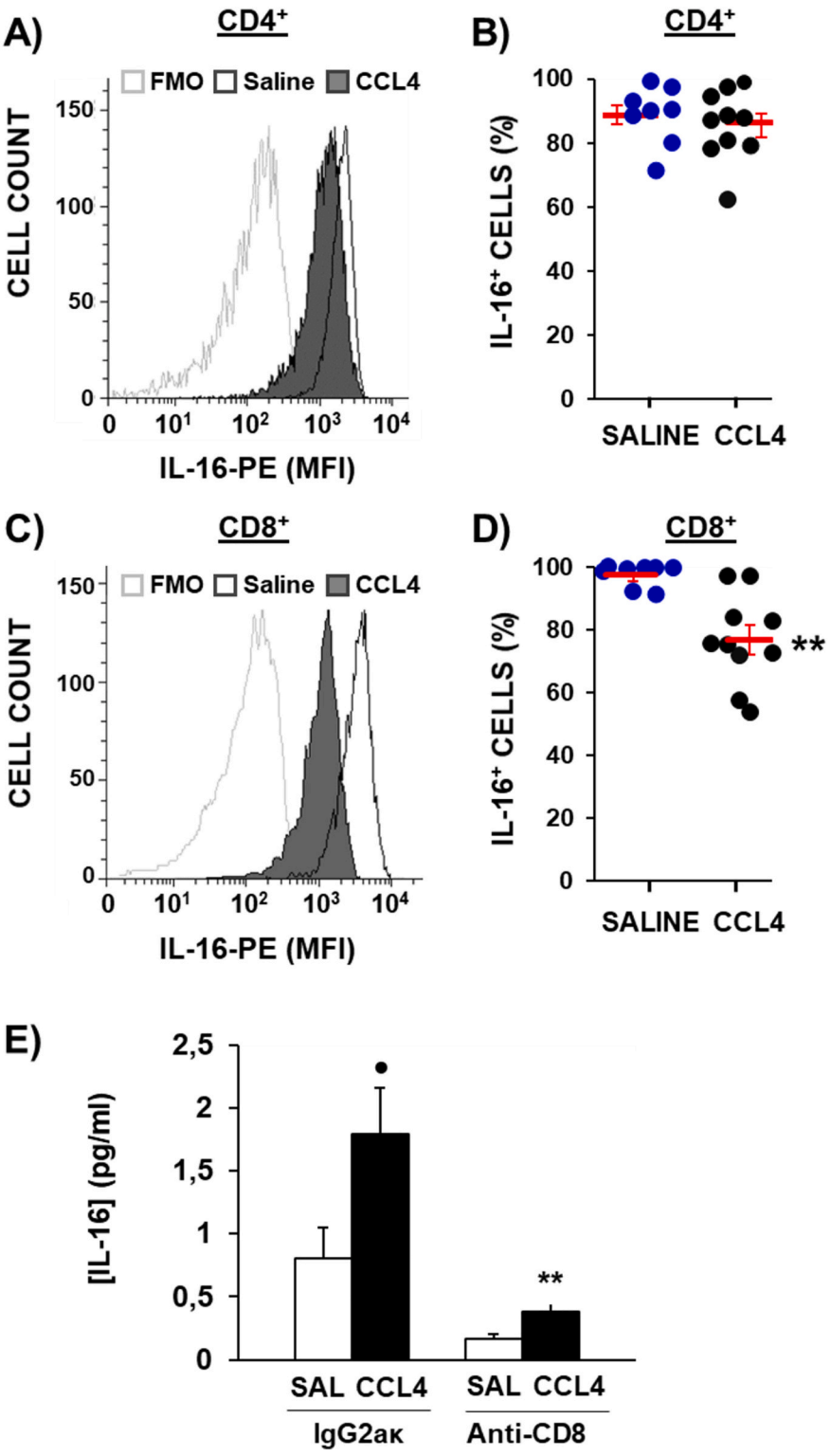

Fig. 7. A, C) Representative histograms of the expression of IL-16 assessed by flow cytometry in CD4+ (A) or CD8+ (C) T-cells of mice s.c. treated 1 h before with saline or 100 ng/kg of CCL4. Data from samples without IL-16 staining (fluorescence minus one, FMO) and their IL-16-stained counterparts are shown. B, D) Percentages of IL-16$^+$CD4$^+$ (B) and IL-16$^+$CD8$^+$ (D) T-cells in mice treated 1 h before with saline ($n = 8$) or 100 ng/kg of CCL4 ($n = 10$). Dots correspond to values obtained in individual mice treated with saline (blue) or CCL4 (black) and red lines represent the mean percentages with their corresponding standard error. $^{**}P < 0.01$ compared with saline-treated mice, Bonferroni's correction.
E) Serum IL-16 levels measured by ELISA in mice i.p. treated 24 h before with an anti-CD8 antibody (20 μg) of the corresponding IgG isotype (IgG2aκ) and receiving the s.c. administration of 100 ng/kg of CCL4 or saline (SAL) 1 h before taking samples. • $P < 0.05$ compared with the corresponding saline-treated group. $^{**}P < 0.01$ comparing both CCL4-treated groups, Bonferroni's correction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a previous report showed that a similar shift from hyperalgesia to analgesia occurred when the hyperalgesic dose of CCL4 is administered to mice in which IL-16 is blocked with a selective antibody [5], it could be proposed that perhaps CD8$^+$ T-lymphocytes could be the main source of IL-16 in response to a high dose of CCL4. This proposition seems compatible with the previously described ability of CD8$^+$ lymphocytes to release IL-16 in a brief period of time (1–4 h) [33] since, due to the constitutive expression of caspase-3, they have a preformed pool of IL-16 that can be immediately released [9]. Thus, trying to determine from which subset of T-lymphocytes IL-16 could be released in response to the high dose of CCL4, the number of CD4$^+$ or CD8$^+$ lymphocytes containing IL-16 was assessed by flow cytometry after treatment with the hyperalgesic dose of CCL4. Whereas the administration of CCL4 did not modify the number of CD4$^+$ T-lymphocytes containing IL-16, it provoked a significant reduction of CD8$^+$ T-lymphocytes expressing IL-16, thus suggesting that this dose of CCL4 is able to evoke the release of

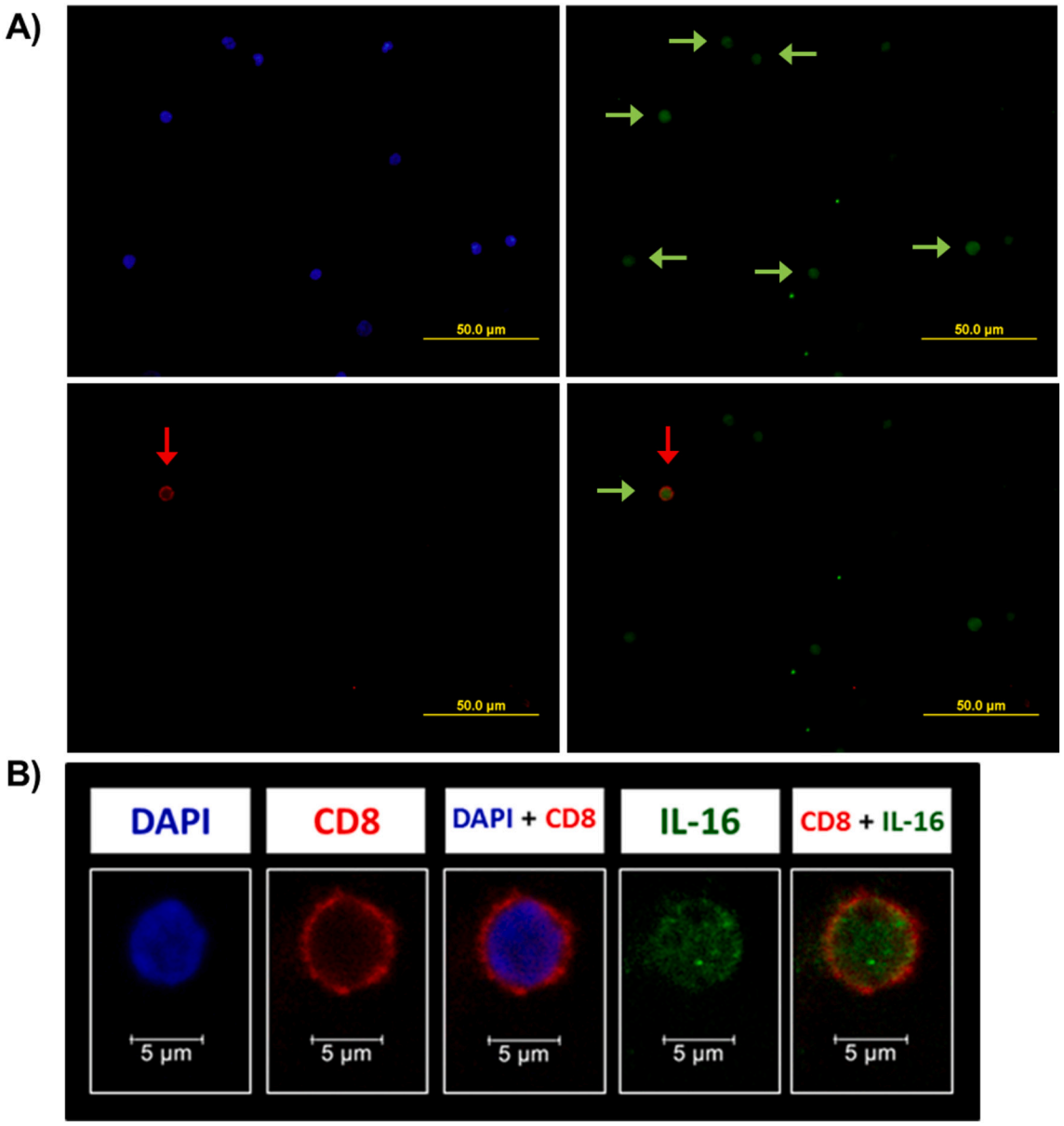

Fig. 8. A) Representative microphotographs corresponding to a double staining immunofluorescence experiment performed on a murine blood smear with an anti-IL-16 antibody (green) and an anti-CD8 antibody (red). Ten cell nuclei were stained with DAPI (blue, upper left image). Six of them were IL-16$^+$ (green, upper right photograph) and only one was CD8$^+$ (red, lower left photograph). The merged image showing positive staining for both antibodies appears in the lower right panel. Green arrows indicate a cell with positive staining for IL-16 and red arrows for CD8.
B) Images from a single IL-16$^+$ CD8$^+$ cell obtained by confocal microscopy showing DAPI, IL-16 and CD8 staining. As can be seen, the green fluorescence produced by the anti-IL-16 antibody was intracellular, whereas the red one produced by the anti-CD8 antibody was located at the membrane level. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

this cytokine from CD8$^+$ T-lymphocytes. Further supporting this possibility, the increase in IL-16 plasmatic levels of observed after the administration of a hyperalgesic dose of CCL4 was completely absent in mice treated with a dose of anti-CD8 antibody able to deplete CD8$^+$ lymphocytes.

5. Conclusions

Overall, these results offer some mechanistic explanation for the role played by IL-16 on the nociceptive effects produced by CCL4 in mice. As previously described, the administration of low doses of CCL4 induces analgesia mediated by met-enk released from CD4+ lymphocytes [3], whereas higher ones lead to hyperalgesic responses, in which IL-16 and other molecules, such as CCL2, IL-1α, CXCL1, CXCL13 participate [5]. As depicted in Fig. 9, the data suggest that hyperalgesic doses of CCL4 provoke the release of IL-16 from CD8$^+$ T-lymphocytes and that this cytokine acts on CD4 receptors expressed in CD4$^+$ lymphocytes leading to CCR5 desensitization. This seems to be a key event to impede met-enk release and favor a hyperalgesic environment orchestrated by the previously mentioned hypernociceptive mediators. Studies in course in our laboratory are designed to try to define if this hypernociceptive role played by IL-16 could be relevant in different pathological settings of experimental pain.

CRediT authorship contribution statement

Sara González-Rodríguez: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Seila**

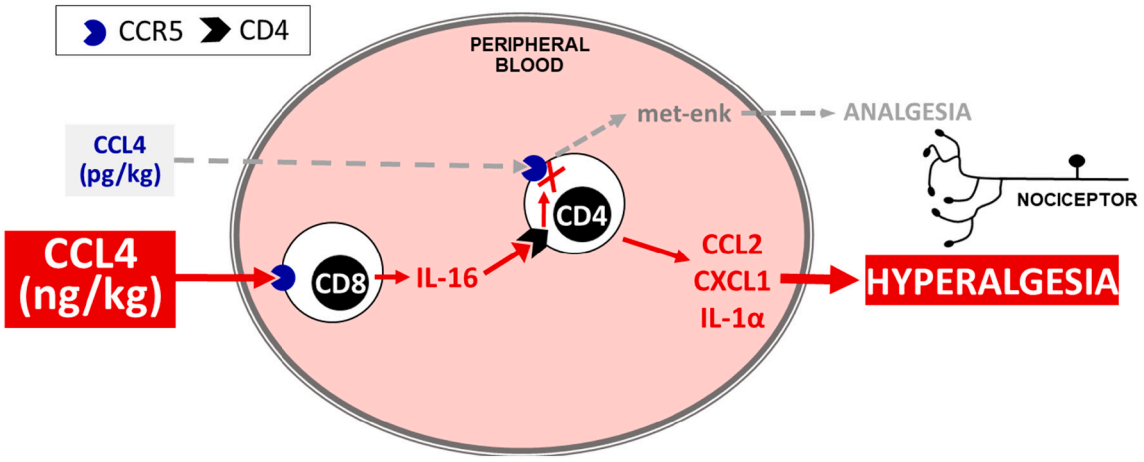

Fig. 9. Graphic explanation of the mechanisms proposed to be involved in the switch from analgesia to hyperalgesia when systemic doses of CCL4 are increased from pg/kg to ng/kg.
The administration of 100 ng/kg of CCL4 leads to CCR5 activation and the subsequent release of IL-16 from circulating $CD8^+$ T-cells. The activation by IL-16 of CD4 receptors expressed in $CD4^+$ T-cells could evoke CCR5 desensitization in this lymphocyte subset, thus impeding the release of met-enk responsible on the analgesia evoked by lower doses of CCL4 [3]. Besides, the blood increase of hypernociceptive mediators, such as CCL2, CXCL1 and IL-1α [5] provokes a hyperalgesic response.

Lorenzo-Herrero: Methodology, Investigation, Writing – original draft. **Christian Sordo-Bahamonde:** Methodology, Investigation. **Agustín Hidalgo:** Supervision. **Segundo González:** Supervision. **Luis Menéndez:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Ana Baamonde:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declared that there is no conflict of interest.

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