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Endogenous Opioid-Mediated Analgesia Is Dependent on Adaptive T Cell Response in Mice

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Pain is an inherent component of inflammation often accompanying immune response. A large spectrum of molecules released within the inflamed tissue induces pain by stimulating primary afferent neurons in situ. Activity of primary sensitive fibers can be counteracted by local opioid release by leukocytes. In this study, we investigated the endogenous regulation of CFA-induced inflammatory pain in the context of adaptive T cell immune response. The nociceptive response to mechanical stimuli was studied using von Frey filaments in mice immunized with OVA in CFA. The nociceptive response of nude versus wild-type mice was dramatically increased, demonstrating T cell deficiency associated with increased pain sensitivity. Based on adoptive transfer experiments of OVA-specific CD4$^+$ T lymphocytes into nude mice, we show that Ag-specific activated, but not resting T lymphocytes are responsible for the spontaneous relief of inflammation-induced pain following Ag challenge. The analgesia was dependent on opioid release by Ag-primed CD4$^+$ T lymphocytes at the inflammatory site. Indeed, T cell-mediated analgesia was inhibited by local injection of an opioid receptor antagonist, unable to cross the blood-brain barrier. Notably, we found opioid precursor mRNA to be >7-fold increased in Ag-specific activated CD4$^+$ T lymphocytes, as compared with resting T lymphocytes in vivo. Taken together, our results show that CD4$^+$ T lymphocytes acquire antinociceptive effector properties when specifically primed by Ag and point out analgesia as a property linked to the effector phase of adaptive T cell response. ***The Journal of Immunology*****, 2011, 186: 5078–5084.**

Painful sensation is a hallmark of the inflammatory response induced by pathogens or tissue damage. A large spectrum of molecules released within the inflamed tissue including neuropeptides, PGs, or proteases induces pain by stimulating primary afferents in situ. Painful messages conveyed by primary sensitive fibers are modulated by peripheral endogenous regulatory mechanisms involving local opioid release by leukocytes infiltrating the inflammatory site. To release opioids and induce analgesia, leukocytes need to be stimulated via activation of cytokine or hormone receptors expressed at their surface (1–3). Indeed, a number of studies suggest that a large part of opioid analgesic effects is dependent on stimulation of opioid receptors expressed on peripheral sensory fibers (4). In early stage of inflammation, both resident cells (i.e., fibroblasts and keratinocytes) and infiltrating inflammatory cells (neutrophils and monocytes) are able to produce endogenous opioids, but not enough to relieve pain. Within the first 96 h of inflammation, leukocyte-mediated analgesia is only observed following injection of triggering factors including IL-1β (5), corticotrophin-releasing factor (1, 5), noradrenaline (6), chemotactic factors (7, 8), and thrombin receptor ligands (9), or exposure to stress such as cold water swim (2, 10). Among immune cells infiltrating the inflammatory site, memory T lymphocytes have been described to be more efficient to relieve pain (5, 11, 12). These findings showing that stimulation of opioid-producing immune cells within inflamed tissues may downmodulate pain perception led us to investigate endogenous regulation of inflammatory pain in the context of adaptive T cell immune response. In addition, although the three classes of endogenous opioids, enkephalins, endorphins, and dynorphins, have been found in leukocytes, their relative prevalence in each immune cell subset has not been investigated to date. Likewise, the nature of leukocytes primarily releasing endogenous opioids in inflammatory states and the immunological status of these cells are still unknown.

In the current study, we show that inflammatory pain relief that occurs spontaneously few days after Ag challenge is caused by opioid release by Ag-primed CD4$^+$ T cells at the site of inflammation. Within draining lymph nodes, specific Ag priming upregulates opioid synthesis in CD4$^+$ T lymphocytes, which, as a consequence, acquire antinociceptive effector properties. Among all the cells involved in adaptive immunity, activated CD4$^+$ Th lymphocytes are the main source of enkephalins, the endogenous opioids predominantly produced by immune cells in mice.

Materials and Methods

Adoptive transfer of anti-OVA TCR-transgenic T cells and immunization of mice

Splenocytes and lymph node cells from DO11.10 BALB/c mice in which >80% of CD4$^+$ T lymphocytes are specific for OVA were pooled. A total of 30×10^6 DO11.10 cells was i.v. injected into syngeneic wild-type or nude BALB/c mice (Charles River Laboratories, Saint Germain sur l'Arbresle, France). The next day, recipient mice were immunized by injecting s.c. into hind footpads 50 μl of either OVA or keyhole limpet hemocyanin (KLH; Sigma-Aldrich, St Louis, MO) emulsified in CFA at 1 μg/μl. To monitor T cell proliferation in vivo, DO11.10 cells were incubated with CFSE (Molecular Probes, Eugene, OR) at room temperature

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Abbreviations used in this article: C_T, cycle threshold; DC, dendritic cell; DOR, δ-opioid receptor; HPRT, hypoxanthine phosphoribosyltransferase; KLH, keyhole limpet hemocyanin; PDYN, prodynorphin; PENK, proenkephalin; POMC, proopiomelanocortin.

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for 10 min and subsequently washed prior to their transfer into recipient mice. CFSE dye dispersing was analyzed by cytofluorometry in anti-OVA TCR-transgenic T cells tracked with the specific anti-clonotypic KJ1-26 mAb (13, 14).

Measurement of nociception

Mechanical withdrawal thresholds were measured using calibrated von Frey filaments of binding forces ranging from 0.04 to 2 g (Stoelting, Wood Dale, IL), applied onto the plantar surface of mice. Ascending series of von Frey filaments were applied, each monofilament being tested five times. Threshold to mechanical stimuli was calculated as the force value of the von Frey filament triggering three paw withdrawals over five applications. Responses to mechanical stimuli were recorded before, and daily after immunization. Naloxone methiodide was injected into the ankle of the inflamed hind paw at a dose of 20 μg in 10 μl PBS, 30 min prior to pain assessment. All experiments involving animals were performed in accordance with ethical guidelines (INSERM) and were approved by the local ethics committee (Midi-Pyrénées, France).

Preparation of immune cell subsets

B lymphocytes as well as $CD4^+$ and $CD8^+$ T lymphocytes were purified from total spleen cells by using cell-negative isolation kits (Invitrogen Dynal AS, Oslo, Norway). $CD4^+$ and $CD8^+$ subsets of T lymphocytes were activated by anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) Abs coated at 2.5 μg/ml in culture dishes for 7 d. B lymphocytes were activated with 20 μg/ml LPS for 18 h. Dendritic cells (DCs) and macrophages were generated from bone marrow cells cultured in RPMI 1640, 10% FCS containing GM-CSF (DCs) or M-CSF (macrophages) for 9 d. Cells were activated by adding 1 μg/ml LPS for the last 18 h of culture.

Cytofluorometric analysis

Cells were incubated with optimal concentrations of Abs for 30 min at 4°C in PBS containing 1% FCS and 2 mM EDTA. The mAbs against mouse cell surface Ags were as follows: anti-CD3 (clone 145-2C11), anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD25 (clone PC61.5), anti-DO11.10 TCR (clone KJ1-26), anti-B220 (clone RA3-6B2), anti-IgM (clone II/41), anti-CD11b (clone M1/70), anti-F4/80 (clone BM8), anti-CD14 (clone rmC5-3), anti-CD11c (clone HL3), anti-CD40 (clone HM40-3), anti-CD86 (clone GL1), and anti-CD69 (clone H1.2F3). All the mAbs were purchased from eBioscience (San Diego, CA). Data were collected on 10,000 living cells by forward and side scatter intensity on a FACS-Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ), and were subsequently analyzed using the Flow Jo software (Tree Star, Ashland, OR).

Immunocytochemistry

Cells were centrifuged onto glass coverslips for 5 min at 750 rpm in a cytospincentrifuge. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed with PBS, and then permeabilized with 0.05% Triton X-100 for 30 min at room temperature. After extensive washing with PBS, cells were incubated with PBS containing 5% FBS for 1 h at room temperature. Rabbit anti–Met-enkephalin polyclonal IgG Abs or normal rabbit control IgG (Chemicon International, Temecula, CA) were then added for 3 h at room temperature. After washing with PBS, cells were incubated with FITC-labeled swine anti-rabbit Fcγ-specific Abs (DakoCytomation, Glostrup, Denmark) for 1 h at room temperature. Fluorescence images were taken using an upright laser scanner confocal microscope (Carl Zeiss MicroImaging GmbH) with ×100 oil immersion objective.

PCR analysis

Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase using random hexamer oligonucleotides for priming. Transcripts encoding for hypoxanthine phosphoribosyltransferase (HPRT), proenkephalin (PENK), proopiomelanocortin (POMC), and prodynorphin (PDYN) were quantified by real-time PCR. Amplification was performed with a Light Cycler 480 (Roche Applied Science, Meylan, France) using SYBR Green I Master (Roche Diagnostics) and the following forward and reverse primers: 5′-GTTCTTTGCTGACCTGCTGGAT-3′ and 5′-CCCC-GTTGACTGATCATTACAG-3′ for HPRT, 5′-CGACATCAATTTCCTG-GCGT-3′ and 5′-AGATCCTTGCAGGTCTCCCA-3′ for PENK, 5′-TGG-CCCTCCTGCTTCAGAC-3′ and 5′-CAGCGAGAGGTCGAGTTTGC-3′ for POMC, and 5′-TGTGTGCAGTGAGGATTCAGG-3′ and 5′-AGACCGT-CAGGGTGAGAAAAGA-3′ for PDYN. The target gene expression was normalized to the HPRT mRNA and quantified relative to a standard cDNA (calibrator sample) prepared from mouse brain, using the $2^{-\Delta\Delta CT}$ method, where ΔΔ cycle threshold (C_T) = $\Delta C_{T\ \text{sample}} - \Delta C_{T\ \text{calibrator}}$. All the primer pairs do not amplify genomic DNA. Sequence analysis of PCR products revealed 100% identity with the corresponding referential cDNA sequence.

Results

T cell deficiency results in an increased sensitivity to inflammatory pain in immunized mice

The role of T cell-mediated immunity in regulation of inflammatory pain was first appreciated by comparing nociceptive response to mechanical stimuli using calibrated von Frey filaments in wild-type and T cell-deficient nude BALB/c mice immunized with OVA emulsified in CFA. Basal mechanical sensitivity measured in contralateral nonimmunized control hindpaws was identical in both groups of mice (Fig. 1). Injection into hind paws of OVA emulsified in CFA resulted in a similar increase in sensitivity to mechanical stimuli in both nude and wild-type BALB/c mice. On day 6 after immunization, CFA-induced hyperalgesia started to decrease in immunocompetent BALB/c mice, whereas it remained unchanged until day 9–10 in T cell-deficient nude BALB/c mice. From days 7 to 10, nociceptive response induced by mechanical stimuli was significantly lower in immunocompetent wild-type BALB/c mice, as compared with T cell-deficient nude BALB/c mice (two-way ANOVA analysis, $F = 22.36$, $p < 0.0001$, OVA-primed wild-type mice versus OVA-primed nude mice). T cell-deficient nude BALB/c mice recovered their basal sensitivity to mechanical stimuli (i.e., similar to that measured in nonimmunized contralateral hind paw) at day 14. CFA-induced inflammatory pain was worsened in Ag-primed immunocompetent BALB/c mice that have been locally administered with naloxone methiodide, an antagonist of the three classes of opioid receptors unable to cross the blood-brain barrier, indicating that the spontaneous antinociceptive activity is mediated through activation of opioid receptors expressed on peripheral afferent sensory neurons (Fig. 1) (two-way ANOVA analysis, $F = 22.15$, $p < 0.0001$, untreated versus naloxone-treated OVA-primed wild-type BALB/c mice). By contrast, mechanical pain sensitivity of OVA-primed nude mice was not altered by naloxone methiodide treatment, indicating that T lymphocytes are the main mediators of opioid-induced analgesia that spontaneously occurred at the sixth day after Ag priming in immunocompetent BALB/c mice (Fig. 1).

$CD4^+$ T lymphocytes specifically primed by Ag induce analgesia via peripheral opioid receptor activation

To determine whether the antinociceptive activity of T cells was an effector property specifically acquired in response to Ag, OVA-specific $CD4^+$ T lymphocytes from DO11.10 mice were passively transferred into syngenic recipient nude mice prior to their immunization with either OVA or KLH in CFA. As shown in Fig. 2, the hypersensitive response to mechanical stimuli was significantly more rapidly reduced in mice immunized with OVA, as compared with those immunized with the irrelevant control Ag KLH (two-way ANOVA analysis, $F = 6.497$, $p = 0.018$, OVA-primed mice versus KLH-primed mice). The T cell-mediated analgesia only observed in mice immunized with relevant Ag was reversed by neutralizing peripheral opioid receptors with naloxone methiodide (Fig. 2).

PENK mRNA is upregulated in Ag-primed $CD4^+$ T lymphocytes in vivo

Because neutralization of analgesia by naloxone methiodide (Fig. 2) suggested a local release of endogenous opioids, we investigated whether opioids were produced by T lymphocytes in re-

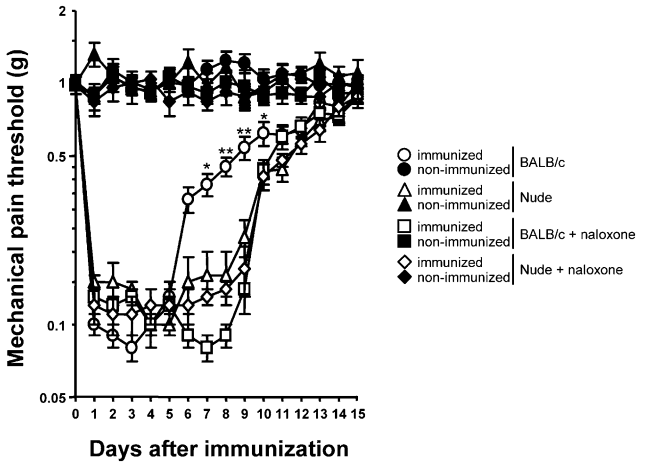

FIGURE 1. Adaptive T cell response concurs to relieve inflammatory pain via peripheral opioid receptor activation. Wild-type (circle and square) and nude (triangle and diamond) mice were immunized s.c. into hind footpads with OVA in CFA. From day 3 until the end of the experiment, mice were daily injected (square and diamond; $n = 12$) or not (circle and triangle; $n = 12$) into inflamed hind paw with naloxone methiodide. Inflammatory pain was monitored by measuring sensitivity of the mice to mechanical stimuli using the von Frey test. Data are expressed as mean ± SEM paw withdrawal thresholds measured in inflamed immunized paws (open symbol) and contralateral noninjected control paws (closed symbol). For each experimental group of mice, the results were obtained from two sets of experiments performed on six mice. Statistical analysis was performed using repeated-measures two-way ANOVA and subsequent Bonferroni posthoc test when appropriate. $*p < 0.05$, $**p < 0.01$ wild-type mice versus nude mice.

sponse to Ag in vivo. Naive T lymphocytes from DO11.10 mice labeled with the fluorescent CFSE dye were passively transferred into syngeneic BALB/c mice prior to their immunization into hind footpads with OVA or KLH emulsified in CFA. Cells were recovered from popliteal and inguinal draining lymph nodes on days 1–3 after Ag priming. As shown by the gradual decrease in CFSE fluorescence intensity, KJ1-26$^+$ anti-OVA T lymphocytes proliferated in response to OVA, but not to KLH (Fig. 3*A*). To compare their ability to produce opioid peptides, KJ1-26$^+$ CD4$^+$ anti-OVA T lymphocytes were isolated from draining lymph nodes of mice immunized with OVA or KLH. Anti-OVA T lymphocytes purified by cytofluorometric cell sorting on day 6 following immunization were 100% pure. mRNA encoding for all three endogenous opioid precursors PENK, POMC, and PDYN was then quantified by real-time PCR. To evaluate the relative ability of the cells to produce each family of opioid precursors, PCR conditions were optimized to amplify each opioid precursor cDNA with an identical efficacy (Fig. 4*A*). Relative mRNA quantification showed that PENK mRNA content is comparatively much higher than POMC and PDYN mRNA in activated CD4$^+$ T lymphocytes (Fig. 4*B*), even though POMC and PDYN mRNA can be detected in saturating PCR conditions (40 cycles) (Fig. 4*C*). These data indicate that enkephalins are the main opioid peptides synthesized by activated T lymphocytes. PENK mRNA level was significantly higher in activated anti-OVA T lymphocytes from mice immunized with OVA than in resting anti-OVA T lymphocytes that have not responded to immunization with KLH ($p < 0.05$; Mann–Whitney U test) (Fig. 3*B*). Thus, PENK mRNA is up-regulated in T lymphocytes upon their Ag-specific activation in vivo. As shown in Fig. 4*D*, Met-enkephalin–containing peptides accumulate within cytoplasm of the cells.

Activated CD4$^+$ T lymphocytes are the main source of immune-derived opioids

We compared the relative ability of the main cellular components of the adaptive immune response to synthesize opioids. The expression level of mRNAs encoding for all three opioid precursors was quantified by real-time PCR in DCs (CD14$^-$/CD11c$^+$) and macrophages (CD14$^+$/CD11b$^+$) derived from bone marrow (>92% pure) and T (CD3$^+$/CD4$^+$ or CD8$^+$) and B (B220$^+$/IgM$^+$) lymphocytes isolated from spleen (>89% pure). LPS-induced activation of DCs, macrophages, and B lymphocytes was monitored, respectively, by the upregulation of CD40 plus CD86, F4/80, and

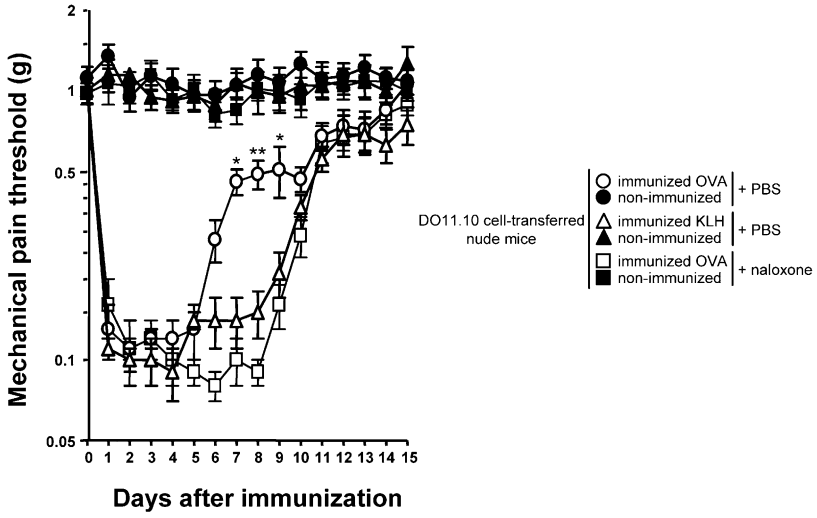

FIGURE 2. Peripheral analgesia is dependent on the specific priming of CD4$^+$ T cells by Ag. Splenocytes and lymph node cells from anti-OVA TCR transgenic DO11.10 mice were transferred i.v. into nonirradiated nude mice. The next day, mice were immunized s.c. into hind footpads with either OVA (circle and square) or KLH (triangle; $n = 12$) in CFA. From day 3 until the end of the experiment, mice immunized with OVA were daily injected (square; $n = 12$) or not (circle, $n = 12$) into hind paw with naloxone methiodide. Inflammatory pain was monitored by measuring sensitivity of the mice to mechanical stimuli using the von Frey test. Data are expressed as mean ± SEM paw withdrawal thresholds measured in inflamed immunized paws (open symbol) and contralateral noninjected control paws (closed symbol). For each experimental group of mice, the results were obtained from two sets of experiments performed on six mice. Statistical analysis was performed using repeated-measures two-way ANOVA and subsequent Bonferroni posthoc test when appropriate. $*p < 0.05$, $**p < 0.01$ OVA-immunized mice versus KLH-immunized mice.

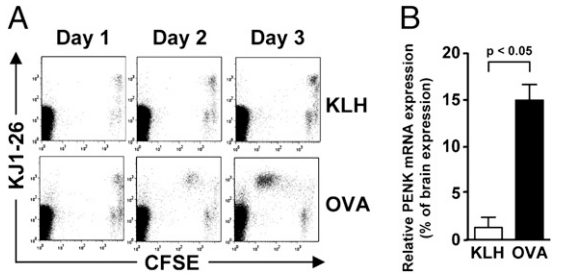

FIGURE 3. PENK mRNA is upregulated in Ag-primed CD4$^+$ T lymphocytes in vivo. *A*, DO11.10 cells labeled with CFSE dye were transferred i.v. into nonirradiated syngeneic BALB/c mice. The next day, mice were primed s.c. into hind footpads with either KLH (*upper panels*) or OVA (*lower panels*) in CFA. Popliteal and inguinal lymph node cells were isolated 1, 2, and 3 d after Ag priming. The figure depicts in the two groups of mice ($n = 9$; 3 mice per day) for each day a representative cytofluorometric analysis of CFSE fluorescence distribution in OVA-specific T lymphocytes stained with anti-TCR KJ1-26 mAb. *B*, Draining lymph nodes were excised from mice on day 6 after priming with KLH (□, $n = 4$) or OVA (■, $n = 4$). CD4$^+$KJ1-26$^+$ lymphocytes were purified from total lymph node cells by cytofluorometric cell sorting using anti-CD4 and anti-TCR KJ1-26 mAbs. PENK mRNA was quantified by real-time quantitative PCR in the anti-OVA CD4$^+$ T cell preparation (100% pure). mRNA content was normalized to the HPRT mRNA and quantified relative to standard mouse brain cDNA using the $2^{-\Delta\Delta CT}$ method. Gene expression in each sample was assessed in three independent experiments run in duplicate. Results (mean ± SEM) are expressed relative to PENK mRNA expression in the mouse brain. Statistical analysis was performed with the Mann–Whitney *U* test.

CD86 at the cell surface. Activation of T lymphocytes by anti-CD3 together with anti-CD28 Abs was checked by the upexpression of CD25 and CD69 molecules (Fig. 5). In our quantitative PCR conditions, PENK, but not POMC and PDYN, was detected, suggesting that PENK-derived enkephalins are the main endogenous opioids produced by immune cells in mice. In resting conditions, PENK mRNA was found in CD4$^+$ T lymphocytes, macrophages, and DCs, whereas it was virtually absent in B and CD8$^+$ T lymphocytes. PENK mRNA was only increased upon activation of DCs and CD4$^+$ or CD8$^+$ T lymphocytes (Fig. 5). PENK mRNA level was significantly higher in activated CD4$^+$ T lymphocytes than in all the other immune cells (one-way ANOVA analysis, $p < 0.001$).

Discussion

Our study highlights analgesia as a property linked to the effector phase of adaptive T cell response and identifies effector CD4$^+$ Th lymphocytes generated in response to Ag as a major source of opioids of hematopoietic origin. Thus, in addition to their effector immune functions devoted to the eradication of pathogens, Ag-primed effector CD4$^+$ T lymphocytes also locally release opioids to relieve inflammatory pain.

Somatic pain induced by immunization with Ag emulsified in CFA more rapidly decreases in wild-type mice than in T cell-deficient nude mice. In agreement with a number of previous studies showing that somatic pain can be counteracted by local opioid release by leukocytes (1, 2, 4), neutralization of opioid receptors in periphery by naloxone methiodide worsened CFA-induced pain in Ag-primed wild-type mice. Thus, analgesia that spontaneously occurs on day 6 after immunization in immunocompetent mice, able to develop an adaptive T cell response to Ag, is mediated through activation of opioid receptors expressed on peripheral afferent sensory neurons.

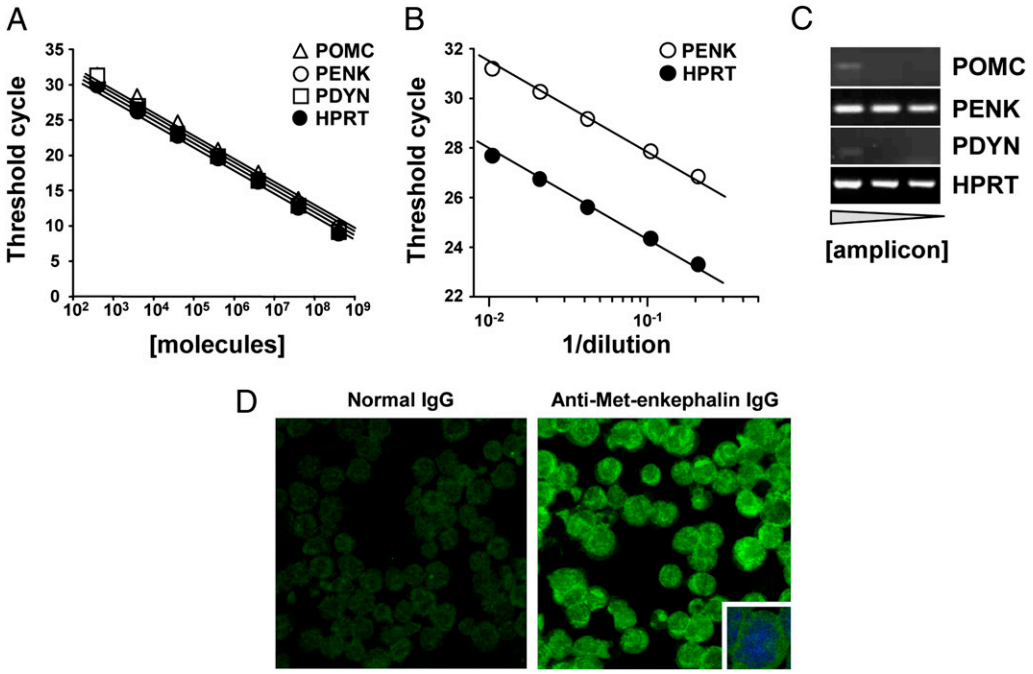

FIGURE 4. Met-enkephalin–containing peptides are highly expressed in Ag-primed CD4$^+$ T lymphocytes in vivo. Opioid precursor mRNA quantification is depicted in *A* and *B*, as follows. *A*, Quantitative PCR standard curves for the POMC, PENK, PDYN, and HPRT genes. For each gene, PCR products that have been previously amplified using specific forward and reverse primers were used as standard cDNA. The figure depicts 10× serial dilution of standard cDNA molecules. Each point of dilution represents the mean of triplicate. *B*, Two-time serial diluted cDNA sample curve. Total RNA extracted from anti-OVA KJ1-26$^+$ CD4 T lymphocytes purified from OVA-primed mice was reverse transcribed and then amplified with specific primers for POMC, PENK, PDYN, and HPRT genes. *C*, PCR products amplified for 40 cycles from cDNA samples diluted 10-fold were run in 2% agarose gel. *D*, Immunochemical detection of Met-enkephalin–containing peptides in anti-OVA KJ1-26$^+$ CD4 T lymphocytes purified from OVA-primed mice. Cells were incubated with either normal control rabbit IgG (*D*, *left panel*) or anti–Met-enkephalin rabbit IgG (*D*, *right panel*). Bound IgG were revealed using FITC-labeled swine anti-rabbit Fcγ-specific Abs. Cell nuclei were stained in blue with DAPI (*inset*). Fluorescence images were acquired by confocal microscopy. Original magnification ×63.

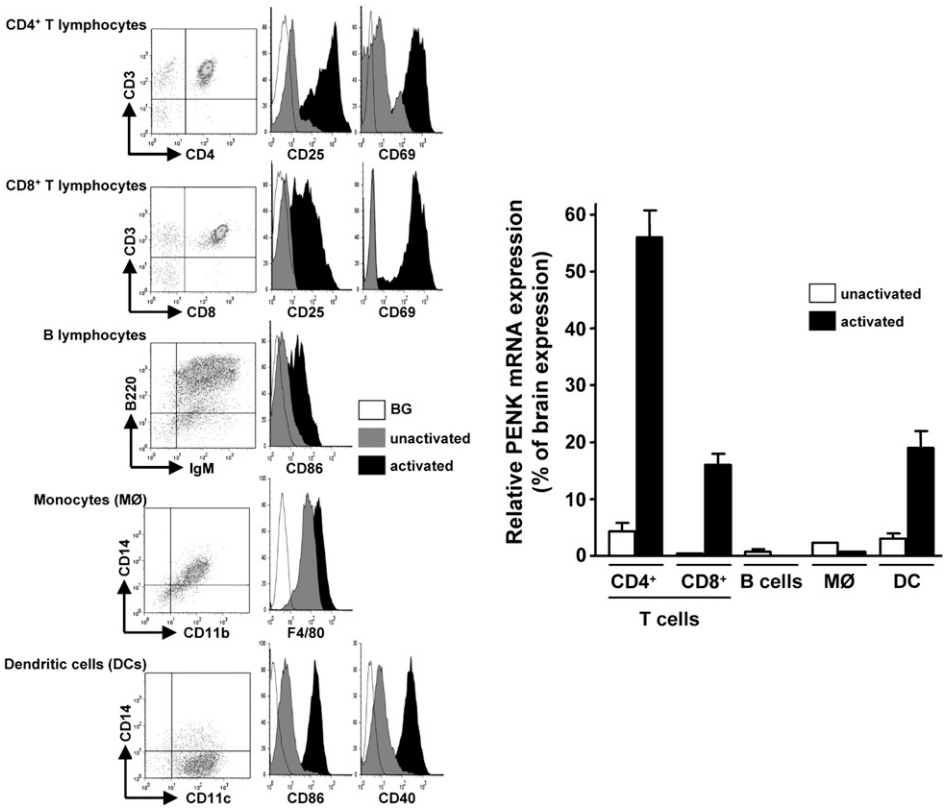

FIGURE 5. PENK mRNA is mainly expressed in activated T lymphocytes and mature DCs. PENK mRNA expression was quantified by real-time PCR in immune cell subsets. $CD4^+$ T lymphocytes ($CD3^+/CD4^+$ double-positive cells), $CD8^+$ T lymphocytes ($CD3^+/CD8^+$ double-positive cells), and B lymphocytes ($B220^+/IgM^+$ double-positive cells) were isolated from spleen of normal BALB/c mice. Macrophages (MØ; $CD14^+/CD11b^+$ double-positive cells) and DCs ($CD14^-/CD11c^+$ positive cells) were derived from bone marrow of normal BALB/c mice. $CD4^+$ T and $CD8^+$ T lymphocytes were activated by using anti-CD3 Abs together with anti-CD28 Abs. B lymphocytes, macrophages, and DCs were stimulated with LPS. The activated status of the cells was testified by the upregulation of CD25 and CD69 on T lymphocytes, CD86 on B lymphocytes and DCs (in addition to CD40), and F4/80 on macrophages. PENK mRNA expression was quantified in unactivated (□) and activated (■) cells. mRNA content was normalized and quantified, as above. Gene expression was assessed in duplicate in at least four independent cell preparations. Data (mean ± SEM) are expressed as percentage of PENK mRNA content in the mouse brain.

Nociceptive response of OVA-primed nude mice remained unchanged following naloxone methiodide treatment. The similar nociceptive behavior of nude mice displaying or not functional peripheral opioid receptors indicates that analgesia occurring a few days after immunization in immunocompetent mice is primarily dependent on T lymphocytes. Interestingly, within the first 4 d following CFA-induced inflammation, the number of opioid receptors expressed on primary sensory neuron terminals increases (15). This increase in the number of opioid receptors together with the disruption of the perineurium and the enhancement of G protein coupling improve the efficacy of opioid peptides (15, 16).

We then determined whether the antinociceptive activity was specifically acquired by T lymphocytes in response to Ag. OVA-specific $CD4^+$ T lymphocytes from DO11.10 mice were passively transferred into nude mice that have been subsequently immunized with either OVA or KLH in CFA. Analgesia was only observed following immunization with OVA, but not KLH, indicating that endogenous opioid-dependent regulation of inflammatory pain is a consequence of the specific T cell response against Ag. The time course of nociceptive sensitivity following immunization with OVA was similar between nude mice restored with anti-OVA $CD4^+$ T lymphocytes and wild-type mice. Indeed, wild-type mice as well as anti-OVA T cell-reconstituted nude mice became less sensitive to nociceptive stimuli on day 6 after OVA priming. This observation is compatible with the course of $CD4^+$ T cell adaptive immune response, including priming of naive $CD4^+$ T cells by Ag-experienced DCs within draining lymph, clonal expansion, and differentiation of Ag-primed T lymphocytes and then migration of differentiated effector memory $CD4^+$ T cells in periphery (17). Thus, pain relief occurring a few days after immunization is dependent on the adaptive $CD4^+$ T cell immune response.

$CD4^+$ T cell-dependent analgesia observed in OVA-primed mice is dependent on peripheral opioid receptors outside the CNS, as the analgesic effect was inhibited by an opioid receptor antagonist that does not cross the blood-brain barrier. The antinociceptive activity associated with the generation of effector $CD4^+$ T lymphocytes in response to Ag is thus elicited by local release of opioids within the inflammatory site. In agreement, PENK mRNA synthesis is >7-fold increased in $CD4^+$ T lymphocytes that have responded to Ag compared with resting $CD4^+$ T lymphocytes that have not responded to Ag in vivo. By contrast, POMC and PDYN mRNA are barely detectable in effector $CD4^+$ T lymphocytes in response to Ag. Thus, the decrease in nociceptive sensitivity that spontaneously occurs a few days after Ag priming is most likely dependent on enkephalin release by Ag-primed $CD4^+$ T lymphocytes.

Exposure to infectious agents induces an early inflammatory reaction causing painful sensation. Inflammatory mediators via DC maturation contribute to initiation of adaptive T cell immune response that in turn will enhance the innate host defense capabilities. In this context, pain could be apprehended as a biological danger

alert signal that will decrease in parallel to the mobilization of Ag-specific effector T lymphocytes within the inflammatory site. Common skin infections such as cellulitis caused by staphylococci or streptococci are often associated with painful sensation. As a matter of fact, pain is a common symptom associated with infections that occurs more often in immunodepressed individuals, including AIDS patients. Given the antinociceptive effect of Ag-specific effector $CD4^+$ T lymphocytes, our study suggests that pain relief, which occurs before the resolution phase of inflammation, is the sign of an active T cell response at the inflammatory site. The role of immune T cell response in other chronic pain situations induced in the absence of infections (i.e., in the absence of CFA) remains to be determined.

The production of opioids has been described in most of the hematopoietic cells (4, 14, 18, 19), but our results, in line with others (5, 11, 12), suggest that effector memory T lymphocytes are more efficient to relieve inflammatory pain. These results suggesting that the potency of immune cells to produce opioids is probably not similar have been testified by quantifying the relative ability of the main cellular components of the adaptive immune response to synthesize all three opioid precursors. In resting conditions, $CD4^+$ T lymphocytes, macrophages, and DCs, but not B lymphocytes and $CD8^+$ T lymphocytes, express PENK mRNA. Cell activation upregulates PENK mRNA in DCs, $CD4^+$ T lymphocytes, and $CD8^+$ T lymphocytes. PENK mRNA level, which is higher in activated $CD4^+$ T lymphocytes than in all other immune cell subsets, may reach >50% of that measured in brain, used as reference. In our quantitative PCR conditions, POMC and PDYN were never detected in all immune cell subsets that have been examined. Thus, in vitro quantification of the relative endogenous opioid content in immune cells confirms that enkephalins are mainly produced by activated $CD4^+$ T lymphocytes that play a major role in endogenous pain regulation.

In line with the predominant production of enkephalins by effector T lymphocytes in vivo, it has been recently shown that δ-type opioid receptor, which exhibits more affinity for enkephalins, plays a major role in relieving CFA-induced inflammatory pain (20). Opioid receptors are expressed in sensory neurons, but also in immune cells, including T lymphocytes and DC. The expression of opioid receptors is not homogeneous among immune cell subsets and may differ according to the local environment and the activation status of the cells (21, 22). δ Opioid receptor (DOR) expressed at a low level in immature DCs and virtually absent in resting T lymphocytes is upregulated upon activation of the cells (23, 24). It has been clearly shown that DOR is upregulated in T lymphocytes upon TCR-mediated activation induced by superantigen administration (25) or Ag priming (14) in vivo. Thus, under inflammatory conditions, the role of DOR is probably not limited to inhibition of pain message. Enkephalins locally released by $CD4^+$ T lymphocytes might also modulate pain-promoting inflammatory response through an auto/paracrine mechanism including downregulation of cytokine production by effector T cells (26).

Taken together, our data show that spontaneous pain relief is a consequence of the specific adaptive T cell-mediated immune response. They also highlight analgesia as a new additional effector function of Ag-experienced $CD4^+$ Th lymphocytes that arrive at the inflammatory site to eliminate pathogens. Opioids are the most efficient drugs to treat pain, but their chronic use may result in side effects including tolerance, respiratory depression, and constipation. Because side effects of opioids are dependent on stimulation of receptors within CNS, the observation that analgesia can be mediated by opioid receptors expressed on peripheral sensory fibers and, therefore, free of side effects has opened new therapeutic perspectives for pain treatment (27). These results highlight $CD4^+$ Th lymphocytes as major actors of endogenous pain modulation in chronic inflammatory disorders. Considering $CD4^+$ Th lymphocytes as cellular targets for the treatment of chronic inflammatory disorders might thus be re-estimated in light of their major role in controlling inflammatory pain.

Disclosures

The authors have no financial conflicts of interest.

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