

μ -Opioid Receptor Is Induced by IL-13 within Lymph Nodes from Patients with Sézary Syndrome

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Endogenous opioid peptides mainly produced by neurons are also released by immune cells. They bind to μ - (μ -opioid receptor, MOR), δ -, and κ -opioid receptors. On the basis of studies on mice showing that MOR is the main mediator of the deleterious effects of opioids on immunity, we wondered whether MOR, absent under normal conditions, is expressed in some pathological situations such as lymphomas. mRNA expression for all three opioid receptors was examined in lymph node biopsy samples from patients with non-Hodgkin's B-cell and T-cell lymphomas. We found that MOR and one of its ligands (enkephalin) are simultaneously expressed almost exclusively in lymph nodes from patients with Sézary cutaneous T cell lymphoma. As MOR was undetectable in circulating malignant T lymphocytes and in normal immune cells, we hypothesized that tumor-released cytokines might induce MOR expression in non-neoplastic lymph node cells. The correlation between mRNA levels of MOR and interleukin-13 (IL-13) within lymph nodes from Sézary patients led us to investigate the ability of IL-13 to upregulate MOR expression in normal immune cell subsets. We found that IL-13 upregulates MOR in activated Langerhans cells. Thus, our data suggest that, under pathological conditions, IL-13 overexpression might allow immune-derived endogenous opioids to down-modulate immune response.

Journal of Investigative Dermatology (2010) **130**, 1337–1344; doi:10.1038/jid.2009.433; published online 28 January 2010

INTRODUCTION

Endogenous opioid peptides belong to three families: endorphins, enkephalins, and dynorphins, which are derived from protein precursors encoded by three distinct genes: the proopiomelanocortin, the proenkephalin, and the prodynorphin genes, respectively. The biological activities of the opioid neuropeptides are mediated by three types of seven-pass transmembrane segment G-protein-coupled receptors named μ - (MOR), δ - (DOR), and κ - (KOR) opioid receptors. β -Endorphin and enkephalins bind to both MOR and DOR, whereas dynorphin interacts only with KOR. The opioid system including endogenous opioid neuropeptides and their receptors are involved in several key neurophysiological functions, such as analgesia, regulation of autonomic nervous

system and neuroendocrine activities, itching, respiration, and gastrointestinal motility. Studies focusing on the therapeutic use of exogenous opioid drugs or the release of endogenous opioids under stressful situations indicated that opioids also affect innate and adaptive immune responses. The link between opioids and immune system is strengthened by the observation that both immune cells and neurons express opioid receptors and have the ability to synthesize endogenous opioid peptides (Labuz *et al.*, 2009).

mRNA encoding opioid receptors have been found in immune cells including T and B lymphocytes, macrophages, and dendritic cells (DCs). The expression level of all three opioid receptor subclasses is not homogeneous among cell subsets and may differ according to the local environment and the activation status. Thus, DOR mRNA is expressed at a low level in immature DCs and is virtually absent in resting T lymphocytes. However, upon activation of the cells, the expression of DOR is upregulated (Nguyen and Miller, 2002; Jaume *et al.*, 2007; Benard *et al.*, 2008). The MOR mRNA undetectable in resting T lymphocytes and found in some activating conditions, is upregulated within an interleukin (IL)-4- or tumor necrosis factor- α -rich micro-environment (Gavériaux *et al.*, 1995; Kraus *et al.*, 2001, 2003a,b; Madden *et al.*, 2001; Jaume *et al.*, 2007; Borner *et al.*, 2008). It is noteworthy that most of the immunosuppressive effects of morphine or stress-induced endogenous opioid release, such as apoptosis of spleen cells, reduction of both inflammation and natural killer cell activity, are

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Abbreviations: DC, dendritic cell; DOR, δ -opioid receptor; KOR, κ -opioid receptor; LC, Langerhans cell; MOR, μ -opioid receptor; MRC1, mannose receptor C type 1; PBMC, peripheral blood mononuclear cells; PENK, proenkephalin; poly(IC), polyinosinic-polycytidylic acid

Received 8 June 2009; revised 24 November 2009; accepted 24 November 2009; published online 28 January 2010

mediated through MOR activation in mice (Shavit *et al.*, 1984; Gavériaux-Ruff *et al.*, 1998; Yin *et al.*, 1999, 2000; Mace *et al.*, 2002; Wang *et al.*, 2002a,b; Philippe *et al.*, 2003). As a matter of fact, although DOR and enkephalins are together expressed in both CD4⁺ T lymphocytes and antigen-presenting mature DCs, opioid receptor neutralization altered neither the antigen-induced proliferation of lymphocytes nor their sensitivity to apoptosis *in vivo* (Jaume *et al.*, 2007).

Considering that MOR is the main mediator of the deleterious effects of opioids on immunity, we wondered whether MOR, which is absent in normal lymph node cells but can be induced by cytokines, is expressed in some pathological situations such as lymphomas. The expression of mRNA encoding for all three opioid receptor classes was examined in a number of lymph node biopsy samples originating from tumor-free patients or from patients with non-Hodgkin's B-cell or T-cell lymphomas. We found that MOR, together with the enkephalin precursor (proenkephalin, PENK), is almost exclusively expressed in lymph nodes from patients with Sézary cutaneous T-cell lymphoma. As both circulating Sézary tumor cells and normal lymph node cells do not express MOR, we hypothesized that tumor-related cytokines might induce MOR expression in surrounding normal immune cells. Our results showed a correlation between mRNA levels of MOR and IL-13 within lymph nodes from Sézary patients. We then investigated the ability of IL-13 to upregulate MOR expression in normal immune cells, including T lymphocytes, monocytes, monocyte-derived conventional DCs or Langerhans cells (LCs). Our results show that MOR is induced by IL-13 in activated LCs, the predominant antigen-presenting cells within lymph nodes draining inflamed skin. On the basis of the MOR-mediated immunosuppressive effects of opioids, our observations provide a new potential opioid-dependent tumor escape mechanism.

RESULTS

MOR is recurrently expressed in lymph node biopsy samples from patients with Sézary cutaneous T-cell lymphoma

Expression of mRNA encoding for PENK and all three opioid receptor subclasses: MOR, DOR, and KOR was investigated by RT-PCR in a variety of lymph node biopsy samples from patients with B-cell and T-cell non-Hodgkin's lymphomas. As shown in Figure 1, the expression of mRNA encoding DOR and KOR was not restricted to a particular group of lymphomas. By contrast MOR mRNA was almost exclusively found in lymph node biopsies from patients with Sézary syndrome. In addition, although DOR and KOR were expressed in about 40% of normal reactive lymph nodes, MOR was not found in physiological conditions (Figure 1). The *PENK* mRNA was expressed in all lymph node specimens, including tumor-free specimens and lymphoma biopsy samples (Figure 1). Sequence analysis of PCR products revealed 100% identity with the corresponding referential complementary DNA sequences (data not shown).

Histiocytes, within lymph nodes from patients with Sézary syndrome, commonly exhibit ceroid deposits accountable for

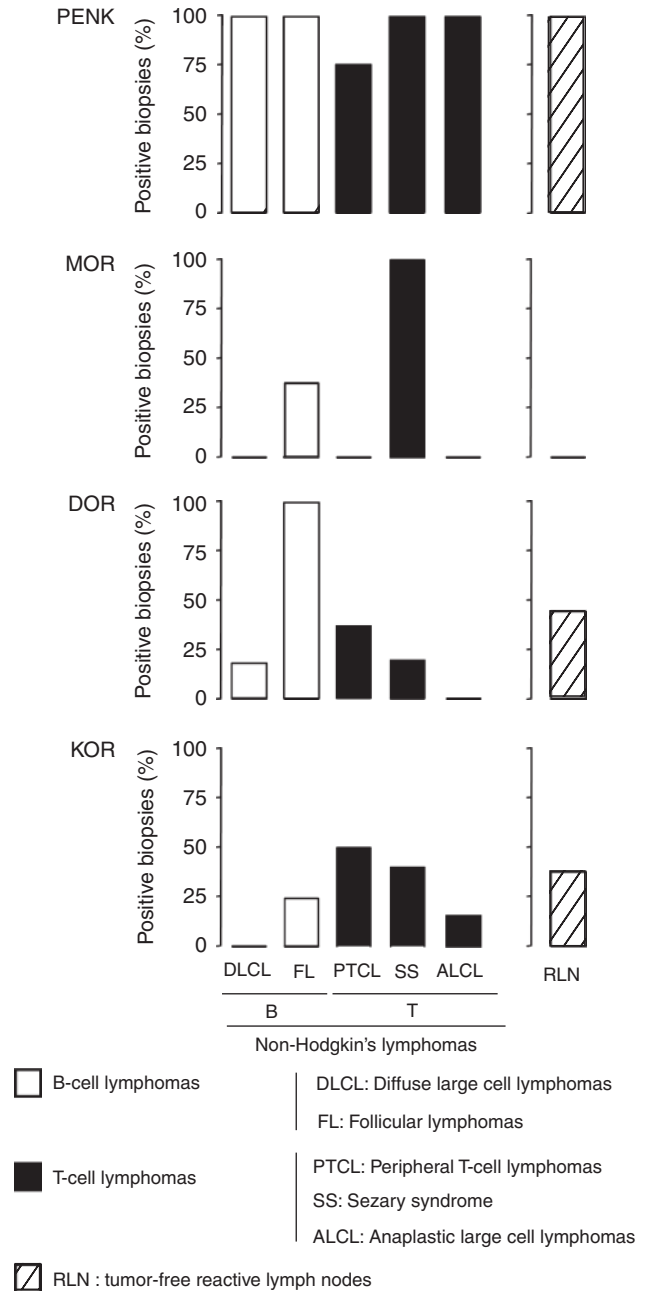


Figure 1. Opioid receptor expression in lymph node biopsies from patients with non-Hodgkin's B- and T-cell lymphomas.

Expression of mRNA encoding for all three opioid receptor classes, MOR, DOR, and KOR, was examined by RT-PCR using the human neuroblastoma cell lines SH-SY5Y and SK-N-MC as controls. cDNA was synthesized from total RNA extracted from tumor-free RLN samples (hatched bars, *n* = 9) and lymph node biopsy samples originating from patients with either non-Hodgkin's B-cell lymphomas (open bars), including DLCL (*n* = 11) and FL (*n* = 8), or non-Hodgkin's T-cell lymphomas (closed bars), including PTCL (*n* = 8), SS (*n* = 5), and ALCL (*n* = 7). RT-PCR experiments were performed twice from total RNA extracted from two independent sets of 20 × 50-μm-thick frozen sections. Results are expressed as percentage of opioid receptor-expressing biopsies. ALCL, anaplastic large cell lymphoma; cDNA, complementary DNA; DLCL, diffuse large cell lymphoma; DOR, δ-opioid receptor; FL, follicular lymphoma; KOR, κ-opioid receptor; MOR, μ-opioid receptor; PTCL, peripheral T-cell lymphoma; RLN, reactive lymph node; SS, Sézary syndrome.

non-specific binding of rabbit IgG. This irrelevant IgG binding excluded the use of commercial polyclonal anti-MOR rabbit antibodies to directly stain MOR-expressing cells *in situ*. Thus, to determine whether MOR expression within lymph nodes from Sézary syndrome patients was due to tumor cells, we took advantage that Sézary syndrome represents the leukemic form of peripheral cutaneous T-cell lymphoma with the presence of large amounts of tumor cells into the blood. The expression of MOR in Sézary tumor cells was investigated by RT-PCR in peripheral blood mononuclear cells containing up to 78% of Sézary cells. The expression of MOR mRNA was never found in peripheral blood leukocytes with a high tumor Sézary cell involvement, as assessed by morphological analysis of blood smears and amplification of CD158k complementary DNA (Ortonne *et al.*, 2006; Table 1).

MOR expression is correlated with that of IL-13 within lymph nodes from patients with Sézary syndrome

The absence of MOR in Sézary tumor cells and lymph node cells under normal conditions (i.e. normal tumor-free reactive lymph nodes) led us to conjecture a key role for the lymph node microenvironment in the acquisition of MOR in Sézary syndrome. As Sézary cells are described as arising from a clonal expansion of mature T helper lymphocytes exhibiting a propensity to produce T helper-2 (Th2)-related cytokines (Dummer *et al.*, 1996), we hypothesized a cytokine-dependent MOR upregulation mechanism in the lymph node microenvironment.

mRNA encoding for the prototypical Th2 cytokines: IL-4, IL-5, IL-10, and IL-13 were quantified by real-time PCR in lymph node biopsies. The mRNA level in each patient's sample was normalized relative to the average of those measured in normal lymph node biopsies. The levels of mRNA for IL-4, IL-5, and IL-10 estimated within lymph nodes

from patients with Sézary syndrome were not significantly different from those measured in tumor-free individuals or in patients with other types of non-Hodgkin's lymphomas (Figure 2). By contrast, IL-13 mRNA expression was significantly higher within lymph nodes from patients with Sézary syndrome as compared with the other groups of patients ($P < 0.001$, one-way analysis of variance, followed by Bonferroni's multiple comparison test). Furthermore, IL-13 mRNA was significantly overexpressed within MOR-expressing lymph node biopsy samples (including MOR-expressing follicular B-cell lymphoma patients) when compared with the

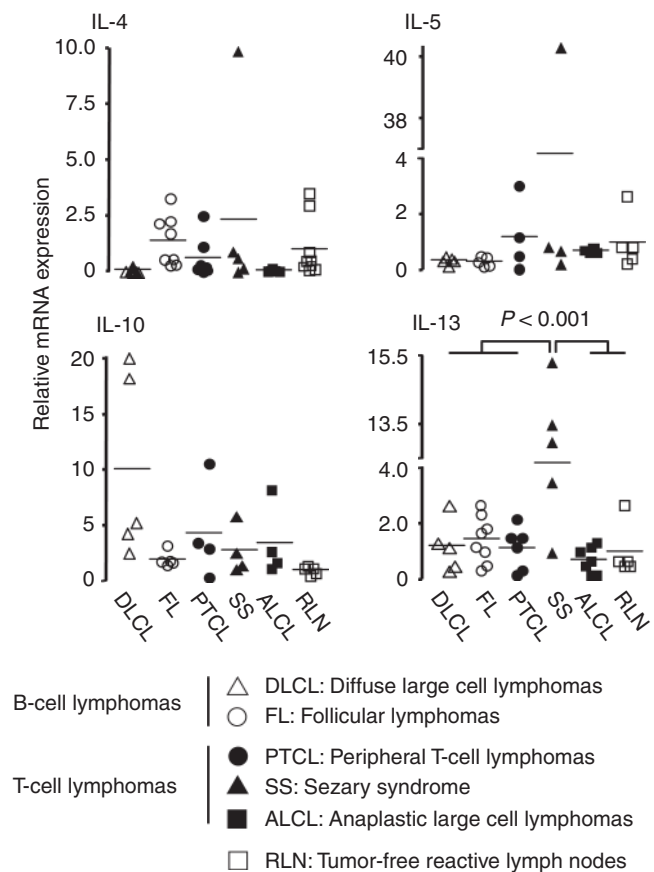


Table 1. MOR expression in PBMCs from patients with Sézary syndrome¹

Patients				
Sex/age (years)	CD3+CD4+ (%)	Sézary cells (%)	CD158K	MOR
M/86	79	26	+	-
M/68	ND	21	+	-
M/51	95	78	+	-
M/39	63	54	+	-
M/49	64	27	+	-
F/54	ND	67	+	-

Abbreviations: CD, cluster of differentiation; MOR, μ -opioid receptor; PBMC, peripheral blood mononuclear cell.

¹PBMCs were isolated from blood samples by density centrifugation. Relative content in CD3⁺CD4⁺ was determined by cytofluorometry. Sézary cells displaying a cerebriform nucleus and a high nucleus-to-cytoplasm ratio were identified through examination of smears. Expression of MOR and CD158k were investigated by RT-PCR.

Figure 2. mRNA expression of the Th2-related cytokines in lymph node biopsies from patients with non-Hodgkin's lymphomas. mRNA encoding for IL-4, IL-5, IL-10, and IL-13 were quantified by real-time PCR in tumor-free RLN (□) and lymph nodes from patients with either non-Hodgkin's B-cell lymphomas, including DLCL (△) and FL (○), or non-Hodgkin's T-cell lymphomas, including PTCL (●), SS (▲), and ALCL (■). mRNA content was normalized to the metastatic lymph node protein 51 (*MLN51*) mRNA and quantified relative to standard cDNA prepared from the Jurkat leukemia T cell line (calibrator sample). For each sample, mRNA level was expressed relative to the average of mRNA levels in normal reactive lymph nodes. Gene expression in each sample was assessed in at least two independent experiments run in duplicate. Data were analyzed by using a one-way ANOVA followed by Bonferroni's multiple comparison test. ALCL, anaplastic large cell lymphoma; ANOVA, analysis of variance; cDNA, complementary DNA; DLCL, diffuse large cell lymphoma; DOR, δ -opioid receptor; FL, follicular lymphoma; IL, interleukin; KOR, κ -opioid receptor; MOR, μ -opioid receptor; PTCL, peripheral T-cell lymphoma; RLN, reactive lymph node; SS, Sézary syndrome.

MOR-lacking biopsy samples ($P < 0.0001$, unpaired t -test), (Figure 3a). The *IL-13* and *MOR* mRNA expression levels were correlated ($P < 0.01$; Spearman correlation test, $r = 0.94$;

Figure 3b). Thus, our data led us to speculate that MOR could be induced by IL-13 in lymph node cells from patients with Sézary syndrome.

MOR is induced by IL-13 in mature LCs

We focused first on the identification of the potential cellular targets of IL-13 by examining the surface expression of the functional IL-13 receptor- $\alpha 1$ chain (IL-13R $\alpha 1$) on immune cell subsets including circulating Sézary tumor cells. The expression of IL-13R $\alpha 1$ was investigated in peripheral blood mononuclear cell gated on CD3 $^-$ CD14 $^+$ monocytes and CD3 $^+$ CD4 $^+$ T lymphocytes from both healthy individuals and Sézary syndrome patients. As expected, the percentage of peripheral blood CD4 $^+$ T lymphocytes was higher in patients than in healthy donors ($77 \pm 14\%$ versus $36 \pm 8\%$, respectively; $P < 0.01$, unpaired t -test) and conversely, the proportion of circulating CD3 $^-$ CD14 $^+$ monocytes was reduced in patients ($6 \pm 5\%$ versus $15 \pm 7\%$; $P < 0.05$, unpaired t -test). In both physiological and pathological conditions, IL-13R $\alpha 1$ was expressed on peripheral blood monocytes but not on lymphocytes (Figure 4). Thus, these observations ruled out a potential effect of IL-13 on Sézary tumor cells. Cell surface expression of IL-13R $\alpha 1$ was further examined on normal immune cell subpopulations under quiescent and stimulated conditions. The activation status of CD4 $^+$ or CD8 $^+$ T lymphocytes, CD3 $^-$ CD14 $^+$ monocytes, and CD14 $^-$ CD1a $^+$ conventional or Langherans DCs was monitored by the upregulation of CD69, *IL-6* mRNA, and

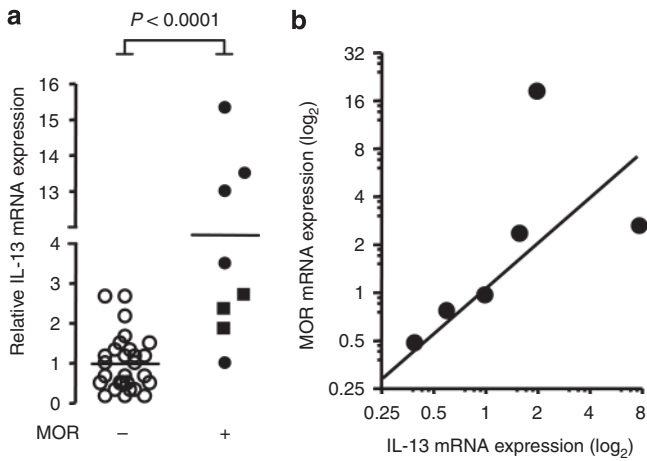


Figure 3. Correlation between MOR and IL-13 mRNA expression in lymph node biopsies. (a) Relative *IL-13* mRNA expression in lymph nodes expressing (+) or not expressing (-) MOR. Lymph node specimens with detectable MOR mRNA included five patients with Sézary syndrome (●) and three patients with follicular B-cell Lymphoma (■). Data were analyzed by using unpaired t -test. (b) Correlation between MOR and *IL-13* mRNA expression was calculated using a non-parametric Spearman correlation test ($r = 0.94$); $P < 0.01$. IL, interleukin; MOR, μ -opioid receptor.

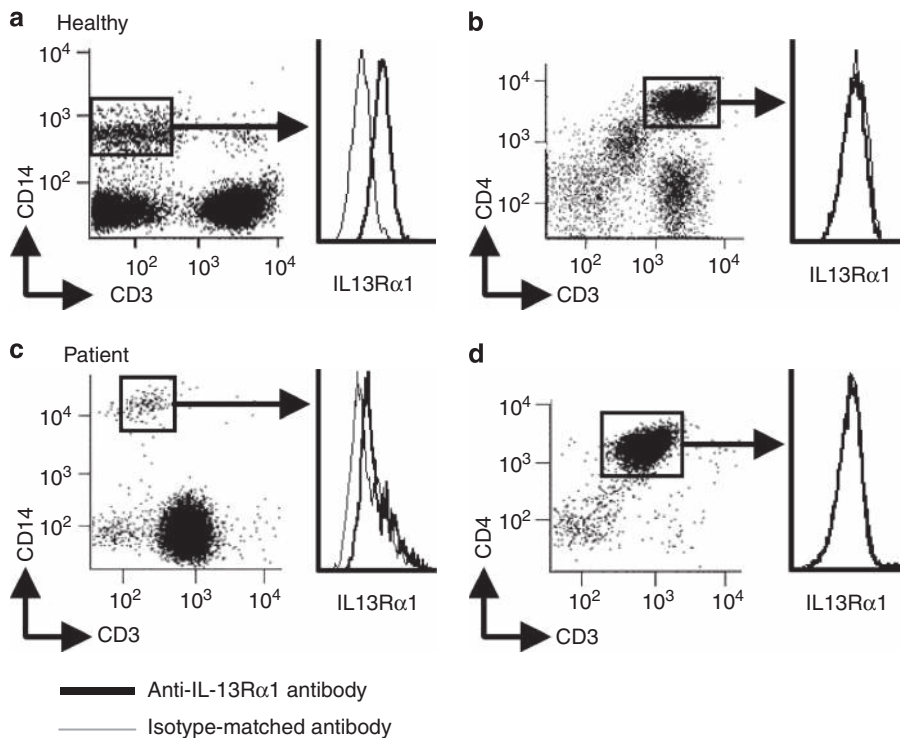


Figure 4. Cytofluorometric analysis of IL-13R $\alpha 1$ expression on peripheral monocytes and lymphocytes from healthy donors and patients with Sézary syndrome. Peripheral blood mononuclear cells, including CD14 $^+$ CD3 $^-$ monocytes (a and c) and CD3 $^+$ CD4 $^+$ T lymphocytes (b and d) from healthy donors (upper panels) and patients with Sézary syndrome (lower panels) were examined for cell surface expression of IL-13R $\alpha 1$. The figure depicts one representative experiment for one healthy blood donor out of seven tested and for one patient with Sézary syndrome out of four. CD, cluster of differentiation.

CD86, respectively (Figure 5). Contrasting with monocytes, IL-13R α 1 was not found on the surface of T lymphocytes, conventional DCs and LCs in resting conditions. Surface expression of IL-13R α 1 was unchanged upon activation of lymphocytes (Figure 5a and b) and conventional DCs

(Figure 5d). However, activation of monocytes (Figure 5c) and LCs (Figure 5e) resulted in an upregulation of IL-13R α 1.

The ability of IL-13 to upregulate MOR in IL-13R α 1-expressing cells was then examined by adding IL-13 to cell culture for 24 hours. Although IL-13 induced IL-13R α 1

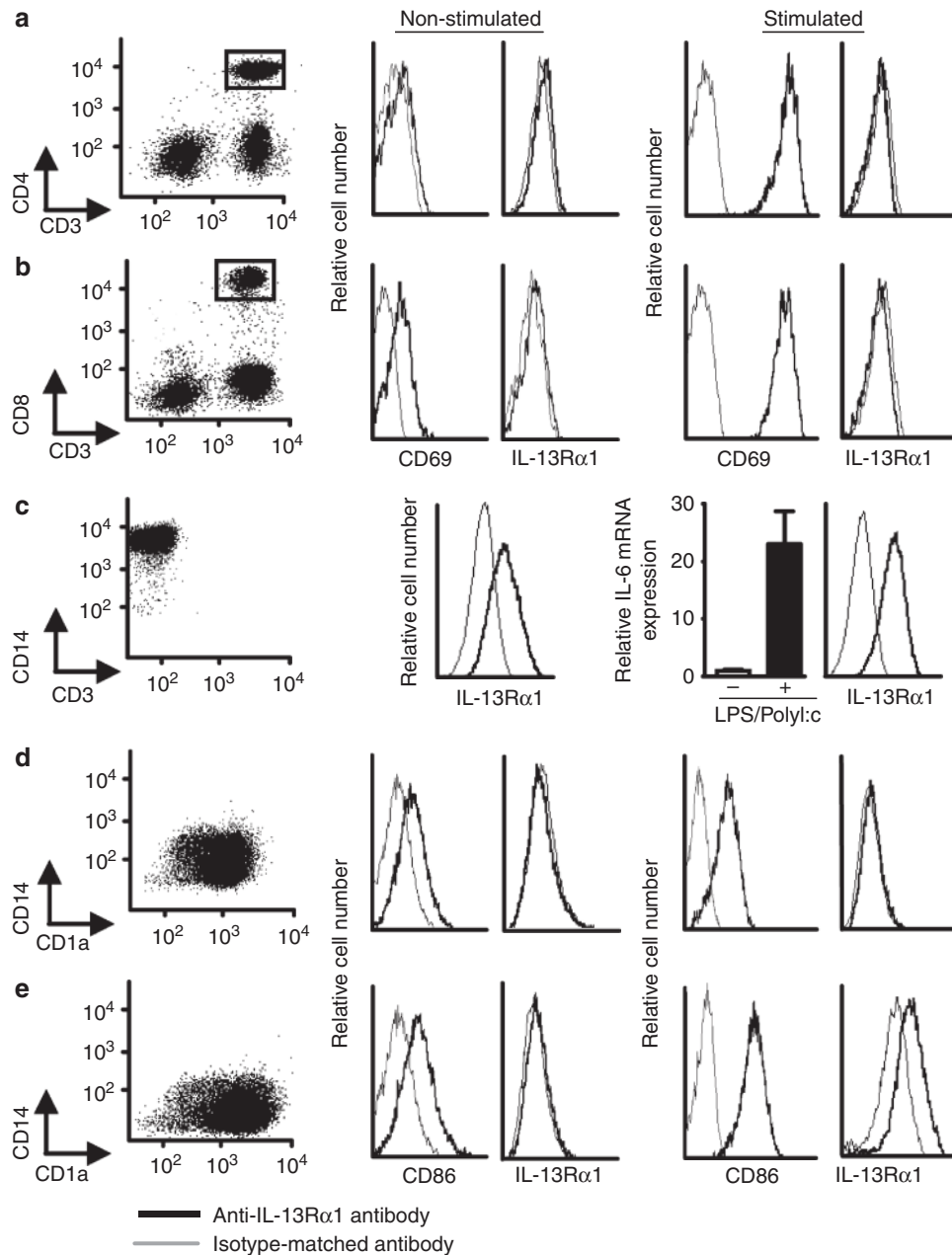


Figure 5. Cytofluorometric analysis of IL-13R α 1 expression on CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes, conventional DCs, LCs, and monocytes from healthy donors. IL-13R α 1 expression was investigated on naive and activated peripheral CD4 $^{+}$ (a) and CD8 $^{+}$ (b) T lymphocytes, on unstimulated and stimulated CD14 $^{+}$ CD3 $^{-}$ monocytes (c) and on immature and mature monocyte-derived CD14 $^{-}$ CD1a $^{+}$ conventional DCs (d) or LCs (e). In (a and b), PBMCs isolated from healthy blood donors were activated (right panels) or not (left panels) by PHA for 24 hours. Cell surface expression of IL-13R α 1 was examined by cytofluorometry in the gated CD4 $^{+}$ (a) or CD8 $^{+}$ (b) T lymphocytes. Cell activation was checked by the expression of the inducible cell surface molecule CD69. In (c), monocytes purified from healthy blood donors by anti-CD14 immuno-magnetic cell sorting were activated (right panels) or not (left panels) by incubating the cells with LPS together with Poly(IC) for 48 hours. Monocyte activation was testified by the increase in *IL-6* mRNA as assessed by real-time quantitative PCR. Conventional (d) and Langerhans (e) DCs were derived from monocytes in the presence of GM-CSF and IL-4 without (d) or with TGF- β (e), respectively. Cell activation (left panels) induced by LPS plus Poly(IC) was assessed by the upregulation of CD86 cell surface expression. Results shown in the figure are representative of at least three independent experiments. CD, cluster of differentiation; DC, dendritic cell; IL, interleukin; LC, Langerhans cell; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; Poly(IC), polyinosinic-polycytidylic acid; TGF- β , transforming growth factor- β .

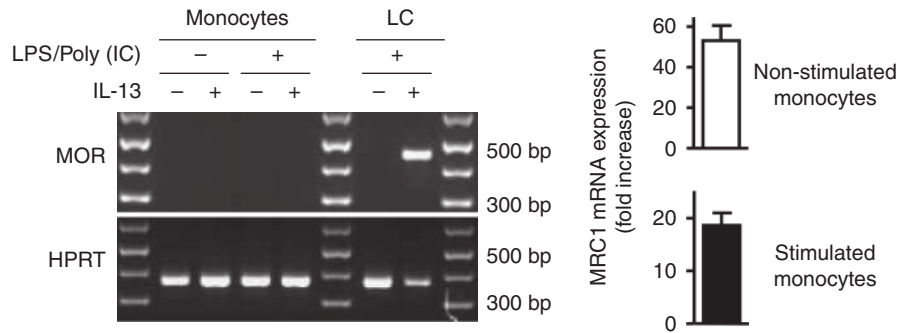


Figure 6. IL-13-induced MOR expression in IL-13R α 1-expressing immune cells. Freshly purified monocytes and monocyte-derived LCs stimulated with LPS plus Poly(IC) were cultured in RPMI 1% FCS with (+) or without (-) 100 ng human rIL-13 for 24 hours. MOR mRNA expression was then examined by RT-PCR as described in Figure 1. Results are representative of three independent experiments. IL-13R α 1 activation by IL-13 in monocytes was evidenced by the increase in MRC1 mRNA as assessed by real-time quantitative PCR (right panels). Results are expressed as mean \pm SD of three independent experiments performed in duplicates. CD, cluster of differentiation; FCS, fetal calf serum; IL, interleukin; LC, Langerhans cell; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; Poly(IC), polyinosinic-polycytidylic acid; MOR, μ -opioid receptor; MRC1, mannose receptor C type 1.

activation both in unstimulated and stimulated monocytes as shown by upregulation of mannose receptor C type 1 and down-modulation of IL-13R α 1 surface expression (data not shown), MOR mRNA remained undetectable. By contrast, IL-13 triggered MOR mRNA expression in activated LCs as assessed by RT-PCR (Figure 6).

DISCUSSION

As shown in experiments comparing wild-type and MOR-deficient mice, most of the immunosuppressive effects of opioids are mediated by MOR (Gavériaux-Ruff *et al.*, 1998; Wang *et al.*, 2002a,b; Philippe *et al.*, 2003). Some of these immunomodulatory effects are dependent on stimulation of MOR *in situ* (Yin *et al.*, 2000; Mace *et al.*, 2002; Wang *et al.*, 2002a,b). However, as exemplified in the T cell response to mitogens, the inhibitory effect of morphine is dependent on the tissue context. The administration of high doses of morphine suppressed the proliferative response of T lymphocytes isolated from spleen but not from lymph nodes (Lysle *et al.*, 1993). In line with these observations, MOR expression in immune lymph node cells, including DCs, macrophages, and lymphocytes is dependent on cytokine environment (Kraus *et al.*, 2001, 2003a,b).

In this study, we found, as previously reported in human peripheral blood mononuclear cells isolated from healthy individuals (Gavériaux *et al.*, 1995), that DOR and KOR, but not MOR, are expressed within normal tumor-free lymph nodes. Whereas most of the lymph node biopsy samples from patients with non-Hodgkin's lymphomas did not express MOR, all Sézary syndrome lymph node samples expressed the receptor. The recurrent expression of MOR was not due to tumor cells colonizing the pathological lymph nodes as assessed by RT-PCR, suggesting a role for the microenvironment in the acquisition of MOR by surrounding normal immune cells. The hypothesis that a cytokine-rich microenvironment might contribute to upregulate MOR expression in normal lymph node cells was supported by the

conjunction of two observations: (1) Sézary syndrome is a neoplastic disorder in which malignant T lymphocytes exhibit a Th2 phenotype (Papadavid *et al.*, 2003), (2) IL-4, the prototypical Th2 cytokine, has been reported to induce MOR mRNA synthesis in normal human blood T lymphocytes and immune cell lines *in vitro* (Kraus *et al.*, 2001). Comparison of the Th2-related cytokine expression within lymph node biopsy samples from patients with non-Hodgkin's lymphomas revealed that the rate of IL-13 mRNA was significantly higher in Sézary syndrome. Several experimental observations argued for a contribution of IL-13 in MOR expression: (1) IL-13 mRNA levels were higher within MOR-expressing lymph nodes than in MOR-lacking lymph nodes, independent of the type of lymphomas, (2) IL-13 and MOR mRNA levels were correlated, and (3) The functional IL-13 receptor, also known as the type II IL-4 receptor, is a heterodimer constituted by the IL-4R α chain and the IL-13R α 1 chain. Thus, as a result of the common IL-4R α chain of their receptors, IL-4 and IL-13 cytokines may share many biological activities (Hershey, 2003).

Surface expression of IL-13R α 1 was then examined on immune cell subsets commonly present within pathological lymph nodes from patients with primary cutaneous T-cell lymphomas. Interleukin-13R α 1 was not found on the surface of malignant CD4⁺ T lymphocytes and on normal CD4⁺ and CD8⁺ T lymphocytes under quiescent or activated conditions (Graber *et al.*, 1998). The inability of IL-13 to stimulate normal and neoplastic lymphocytes pointed out an IL-13-mediated upregulation of MOR in surrounding normal immune cells other than lymphocytes. In the absence of Toll-like receptor-mediated stimulation, IL-13R α 1 was found on monocytes but not on conventional DCs or LCs. Owing to the low reactivity of LCs to Toll-like receptor 4 ligand (van der Aar *et al.*, 2007), we used a combination of lipopolysaccharide and polyinosinic-polycytidylic acid to stimulate monocytes and both DC subsets. Although IL-13R α 1 remained undetectable in mature conventional DCs, the

increase in IL-13R α 1 on both LCs and monocytes after Toll-like receptor -activation led us to consider them as two potential targets for IL-13.

The capacity of IL-13 to induce MOR expression was assessed on IL-13R α 1-expressing cells, including monocytes and activated LCs. Interleukin-13-induced signaling is mainly mediated through IL-4R α chain (Hershey, 2003) and results in the activation of signal transducer and activator of transcription 6 (STAT6) that has been reported to directly bind to MOR promoter (Kraus *et al.*, 2001). As shown, IL-4-responsive, STAT6-binding sites might also mediate IL-13-induced MOR transcription (Kraus *et al.*, 2003a,b). However, we showed that IL-13 does not upregulate MOR mRNAs in monocytes within 24 hours. Thus, IL-4-mediated MOR upregulation in monocytes could be rather mediated through type I IL-4 receptor (IL-4R α /common γ -chain) complex, although we can not formally rule out that an IL13-dependent MOR upregulation in monocytes requires additional protein synthesis and occurs later than 24 hours (not investigated for technical reasons). Response to IL-13 may differ between cell types. As matter of fact, it has been reported that lipopolysaccharide-induced tumor necrosis factor- α production was efficiently inhibited in monocytes but not in macrophages (Hart *et al.*, 1999). Similarly, IL-13 triggered an increase in MOR mRNA content in activated LCs but not in monocytes, whereas they reacted to IL-13 by upregulating MRC1 (mannose receptor C type 1) mRNA. Taken together, our data suggest that MOR expression is dependent on IL-13, as well as subset and activation status of DCs within lymph nodes.

Tumor microenvironment may generate permissive conditions favoring neoplastic cell growth by inhibiting activation and/or effector phases of the immune response (Rabinovich *et al.*, 2007). Considering that MOR is the main mediator of the immunosuppressive effects of opioids, our results suggest that endogenous opioids produced within Sézary lymph nodes might participate in the escape of malignant cells from the immune response. Itching is a feature of Sézary syndrome. The physiopathological mechanisms of peripheral itching are not clearly understood but the role of opioids is well accepted (Bigliardi *et al.*, 2009). Thus, an IL-13-rich environment might also favor an opioid-mediated itching by upregulating MOR on cutaneous afferent sensitive fibers in damaged skin.

MATERIALS AND METHODS

Biopsy specimens

Frozen lymph node biopsies from 39 patients with non-Hodgkin's lymphomas, including 11 patients with diffuse large cell lymphoma, 8 patients with follicular lymphoma, 8 patients with unspecified peripheral T-cell lymphoma, 5 patients with Sézary syndrome, and 7 patients with anaplastic large cell lymphoma were obtained from our tissue bank (Department of Pathology, CHU Purpan, Toulouse, France). Biopsies from nine tumor-free reactive lymph nodes were also analyzed. According to the Declaration of Helsinki, a formal consent was signed by all patients included in the study. Institutional ethical approval was obtained in compliance with the Helsinki agreement from "comité consultatif de protection des personnes et des biens (CCPPB)" at the Purpan Hospital, Toulouse France.

Cytofluorometric analysis

Cells were incubated with optimal concentrations of antibodies for 30 minutes at 4 °C in phosphate-buffered saline containing 5% fetal calf serum, 5 mM EDTA and 0.1% sodium azide. The mouse anti-human monoclonal antibodies against surface antigens used were: anti-CD3 (clone UCHT1), anti-CD4 (clone 13B8.2), anti-CD8 (clone SFC121Thy2D3), anti-CD14 (clone RMO52), anti-CD69 (clone TP1.55.3) from Beckman Coulter (Fullerton, CA), anti-CD14 (clone M5E2), anti-CD86 (clone 2331 (FUN-1)), anti-CD1a (clone HI149), anti-E-Cadherin (clone 36/E-Cadherin) from BD PharMingen (BD Biosciences, San Jose, CA) and anti-IL-13 receptor α 1 chain (IL-13R α 1; clone 419718; R&D systems, Minneapolis, MN). Data were collected on 10,000 living cells by forward and side scatter intensity on an Becton Dickinson LSR II flow cytometer and were subsequently analyzed using the BD FACS Diva Software (Becton Dickinson).

PCR analysis

Total RNA was isolated from tissue samples by guanidine isothiocyanate-phenol-chloroform extraction using ready-to-use TRIzol Reagent (Invitrogen, Carlsbad, CA). The RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) using random hexamer oligonucleotides for priming. In negative controls, the reverse transcriptase was replaced by H₂O. complementary DNA encoding for human MOR, DOR, KOR, CD158k, and hypoxanthine phosphoribosyl transferase were amplified by PCR as previously described (Jaume *et al.*, 2004) using primers listed in Supplementary Table S1 online. All the primer pairs used do not amplify genomic DNA.

The mRNAs encoding for IL-4, IL-5, IL-6, IL-10, IL-13, PENK, MOR, and mannose receptor C type 1 were quantified by real-time PCR. Primers were designed by using Primer Express Software (Applied Biosystems, Foster City, CA; Supplementary Table S2). The amplification was performed with an ABI Prism 7000 Sequence Detector (Applied Biosystems) using the PCR SYBR Green sequence detection system (Eurogentec, Seraing, Belgium). The PCR conditions were: 2 minutes at 50 °C, 10 minutes at 95 °C followed by 40 cycles of two steps including denaturation: 15 seconds at 94 °C and primer annealing/extension: 1 minutes at 60 °C. Data were analyzed using the software supplied with the Sequence Detector (Applied Biosystems). The mRNA content was normalized to the metastatic lymph node protein 51 (*MLN51*) mRNA and quantified relative to a standard complementary DNA (calibrator sample) prepared from the human Jurkat leukemia T cell line or the human monocytic cell line U937 for cytokines, and from the human neuroblastoma cell lines SH-SY5Y and SK-N-MC for MOR and PENK, respectively, using the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = \Delta C_{T \text{ sample}} - \Delta C_{T \text{ calibrator}}$. Gene expression in each sample was assessed in at least two independent experiments run in duplicates. All the primer pairs used do not amplify genomic DNA.

Cells and culture conditions

Peripheral blood mononuclear cells were prepared from freshly heparinized blood samples or buffy coats by density gradient centrifugation in Ficoll-Plaque (Amersham, GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). The CD14-expressing monocytes were positively selected from peripheral blood mononuclear cells by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Bergish Gladbach, Germany). Preparations of

monocytes were more than 93% pure as assessed by cytofluorometry. Conventional DCs were generated from monocytes in RPMI 10% fetal calf serum containing 100 ng ml⁻¹ (1,000 U ml⁻¹) of human recombinant GM-CSF (PeproTech, Rocky Hill, NJ) and 50 ng ml⁻¹ (250 U ml⁻¹) of human recombinant IL-4 (PeproTech). Langerhans cells were generated from monocytes by using GM-CSF, IL-4, and 10 ng ml⁻¹ (200 U ml⁻¹) of human recombinant transforming growth factor 1 (Geissmann *et al.*, 1998). After 6 days of culture, nonadherent cells were collected. Preparations were more than 90% DC-enriched as assessed by cytofluorometry. Phenotype of LCs was monitored by E-Cadherin upregulation as compared with conventional DCs (Geissmann *et al.*, 1998). T cells were nonspecifically activated by adding 3 µg ml⁻¹ phytohemagglutinin (Murex Diagnostics Limited, Dartford, UK) for 24 hours. Activation of monocytes and that of conventional DCs and LCs was induced by adding 1 µg ml⁻¹ lipopolysaccharide together with polyinosinic-polycytidylic acid (10 µg per 5 × 10⁵ cells; Sigma, St Louis, MO) for 48 hours. For cytokine stimulation, after washing with phosphate-buffered saline, cells were cultured in RPMI 1% fetal calf serum (Kraus *et al.*, 2001) to reduce interference with growth factors or cytokines already present in the serum.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr S Fruchon and Dr L Ysebaert for technical support and advice. This study was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), Université Paul Sabatier, Toulouse III, Centre National de la Recherche Scientifique (CNRS), Université Pierre et Marie Curie Paris VI, la Ligue contre le Cancer comités du Tarn et Garonne et du Gers. AB was supported by fellowships from Fondation de la Recherche Médicale and Association pour la Recherche sur le Cancer.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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