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RESEARCH PAPER

Depletion and recovery of lymphoid subsets following morphine administration

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BACKGROUND AND PURPOSE
Opioid use and abuse has been linked to significant immunosuppression, which has been attributed, in part, to drug-induced depletion of lymphocytes. We sought to define the mechanisms by which lymphocyte populations are depleted and recover following morphine treatment in mice.

EXPERIMENTAL APPROACH
Mice were implanted with morphine pellets and B- and T-cell subsets in the bone marrow, thymus, spleen and lymph nodes were analysed at various time points. We also examined the effects of morphine on T-cell development using an *ex vivo* assay.

KEY RESULTS
The lymphocyte populations most susceptible to morphine-induced depletion were the precursor cells undergoing selection. As the lymphocytes recovered, more lymphocyte precursors proliferated than in control mice. In addition, peripheral T-cells displayed evidence that they had undergone homeostatic proliferation during the recovery phase of the experiments.

CONCLUSIONS AND IMPLICATIONS
The recovery of lymphocytes following morphine-induced depletion occurred in the presence of morphine and via increased proliferation of lymphoid precursors and homeostatic proliferation of T-cells.

LINKED ARTICLE
This article is commented on by Eisenstein, pp. 1826–1828 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2011.01513.x

Abbreviations
DN, double negative; DP, double positive; FTOC, fetal thymic organ culture; ISP, immature single positive; SP, single positive; TCR, T-cell receptor; TSP, transitional single positive

Introduction

Opioid use and abuse renders individuals susceptible to infection (see Eisenstein *et al.*, 2006; Wang *et al.*, 2008) and a variety of mechanisms have been proposed to explain how opioids suppress the immune system. These mechanisms include effects on both the innate and adaptive branches of the immune system. Within the adaptive immune system, morphine treatment in mice has been demonstrated to induce profound loss in thymic and splenic mass, but the lymphoid tissues recover over time (Bryant *et al.*, 1987; Bryant *et al.*, 1988a; Sei *et al.*, 1991; Freier and Fuchs, 1993). In addition to inducing lymphocyte depletion, morphine can also alter lymphocyte function (Odunayo *et al.*, 2010). Our goal here was to determine the mechanisms by which the lymphocyte populations recovered after depletion and whether this recovery could take place while serum morphine levels remained at physiologically significant levels. By understanding these mechanisms, we will be able to understand how morphine affects immunity and develop strategies to avoid the detrimental effects of morphine.

To determine the mechanisms of recovery, the B- and T-cell populations that remain after morphine treatment must first be characterized. Although it is known that morphine treatment can deplete total B and T-cells, the subpopulations of lymphocytes that remain after morphine treatment are not defined, especially for B-cells.

Lymphoid development is characterized by an ordered set of steps that result in fully functional mature B- and T-cell subsets. For B-cell development, the first stage in which committed B-cell precursors can be identified in the bone marrow is the pro-B-cell stage (Hardy and Hayakawa, 2001). During this stage, rearrangements in the μ heavy chain genomic locus begin. Upon expression of the μ chain, the cells enter the pre-B-cell stage. Then, cells rearrange the light chain genomic loci and become immature B-cells. Some immature B-cells migrate to the spleen where they can be identified as transitional stage 1 (T1) B-cells (Chung *et al.*, 2003). T1 cells differentiate into transitional stage 2 (T2) cells and ultimately become either follicular (FO) B-cells or marginal zone (MZ) B-cells. The effects of morphine treatment on these B-cell subsets have not been defined.

Like B-cell development, T-cell development proceeds in an ordered manner. The earliest T-cell precursors identified within the thymus lack CD4 and CD8 expression and are called $CD4^{-}CD8^{-}$ double negative (DN) cells. DN thymocytes can be divided into the DN1 ($CD44^{hi}CD25^{-}$), DN2 ($CD44^{hi}CD25^{+}$), DN3E ($CD44^{lo}CD25^{hi}$), DN3L ($CD44^{lo}CD25^{lo}$) and DN4 ($CD44^{lo}CD25^{-}$) subsets (Godfrey *et al.*, 1993; Zeng *et al.*, 2007). During the DN1 and DN2 stages, cells receive signals that induce commitment to the T-cell lineage and begin rearrangement of the genomic locus that encodes the T-cell receptor (TCR) β chain. TCRβ protein can be first detected at the DN3E stage of development; approximately 20% of DN3E thymocytes express TCRβ protein. Upon expression of TCRβ, DN thymocytes proliferate and differentiate through the DN3L and DN4 stages. After the DN4 stage, cells express CD8 and become immature single positive (ISP) $CD8^{+}$ T-cells before expressing CD4 and becoming double positive (DP) thymocytes. During the DP stage, cells rearrange the genomic locus encoding TCRα, express TCRα protein, and express a complete TCR complex. Once the TCR is expressed, positive and negative selection occur, the processes by which the T-cell repertoire is selected. Some DP thymocytes down-regulate CD8 to become transitional single positive (TSP) $CD4^{+}$ thymocytes, which then mature into single positive (SP) $CD4^{+}$ and SP $CD8^{+}$ thymocytes (Lundberg *et al.*, 1995; Suzuki *et al.*, 1995; Lucas and Germain, 1996; Dalheimer *et al.*, 2009). Previous analyses of the thymocyte populations in morphine-treated mice demonstrated that DP thymocytes are highly susceptible to morphine treatment (Sei *et al.*, 1991; Freier and Fuchs, 1993). However, the effects of morphine on the DN and SP subsets have not been investigated.

In this report, we have defined the B- and T-cell subsets that remain when mice are treated with morphine and examine the mechanisms by which B and T-cells repopulate the primary and secondary lymphoid organs. In particular, we studied the production of lymphocytes in primary lymphoid organs and homeostatic proliferation of cells in secondary lymphoid organs. We demonstrate that morphine-induced corticosteroid production was the most likely cause of depletion of immature lymphocytes. After depletion, B- and T-cell subsets recovered via a combination of increased proliferation of lymphoid precursors and homeostatic proliferation of mature T-cells.

Methods

Mice

All animal care and experimental protocols complied with and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Male C57BL/6 mice were housed and bred under specific pathogen-free conditions. At the onset of the experiments, mice were between the ages of 8 and 12 weeks.

Pellet implantation

Mice were anesthetized with ketamine (75 $mg \cdot kg^{-1}$) and xylazine (7.5 $mg \cdot kg^{-1}$) and pellets were implanted subcutaneously. Morphine 75 mg, naltrexone 30 mg, and placebo pellets were obtained through the NIH AIDS Research and Reference Program, Division of AIDS, NIAID, NIH. Tissues were harvested on days 7, 14 and 21 post-implantation.

Antibodies

Anti-CD3-PE-Cy7, anti-CD8α-Alexa Fluor 647, anti-CD4-FITC, anti-CD44-PE-Cy7, anti-CD44-Horizon V450, anti-CD25-APC-Cy7, anti-CD24-PE-Cy7, anti-TCRβ-PE, anti-B220-PE, anti-IgM-FITC, anti-CD34-Alexa Fluor 647, anti-IgD-Alexa Fluor 647, anti-CD21-PE-Cy7, anti-CD23-Pacific blue and anti-CD62L-PE-Cy7 were purchased from BD Biosciences (San Jose, CA, USA), eBiosciences (San Diego, CA, USA) or Biolegend (San Diego, CA, USA).

Cell labelling and flow cytometry

Single cell suspensions of thymocytes, splenocytes and lymphocytes were collected by gently disrupting the tissue using a wire mesh and a syringe plunger. Bone marrow cells were collected by flushing one femur with PBS using a 27-gauge needle. Cells were filtered through a 100 μm nylon mesh. Surface, intracellular staining and DNA staining were performed as described earlier (Zeng *et al.*, 2007). Briefly, cells were surface labelled by incubating cells with antibodies for 30 min on ice in staining buffer [PBS containing 2% Fetal Clone I (HyClone Laboratories, Inc., Logan, UT, USA)]. After washing, cells were fixed in 1% paraformaldehyde. For intracellular staining, fixed cells were permeabilized and labelled in permeabilization buffer (0.3% Tween-20 in PBS with 2% Fetal Clone I). For DNA staining, fixed cells were incubated with 1 $\mu g \cdot mL^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI) in permeabilization buffer for 30 min and analysed immediately. For cell cycle analysis, single flow cytometric events were defined using DAPI-Area versus DAPI-Height and cells in the S, G2 or M phase of the cell cycles were defined as those cells that contained greater than 2N DNA. Samples were analysed using a BD LSRII (BD Biosciences, San Jose, CA, USA). Data were analysed using the BD FACSDiva software (BD Biosciences) or FlowJo (TreeStar, Inc., Ashland, OR, USA).

Serum morphine concentrations

To 10 μL serum, the internal standard, d3-morphine, was added. Protein was removed by extracting the morphine into

Table 1

Spleen, lymph node, bone marrow and thymus cell numbers in morphine-treated and control groups

	Morphine (n = 13)	Morphine + naltrexone (n = 7)	Naltrexone (n = 7)	Placebo (n = 7)
Spleen				
B cells	6.9 ± 7.2	14 ± 7.7	14 ± 7.2*	18 ± 15*
$CD4^+$ cells	2.3 ± 1.1	6.1 ± 1.8*	6.2 ± 2.0*	6.0 ± 3.5*
$CD8^+$ cells	2.1 ± 0.91	3.9 ± 0.85*	3.8 ± 1.1*	5.7 ± 4.3*
Lymph nodes				
B cells	1.2 ± 0.96	7.5 ± 3.1	2.7 ± 1.4	2.4 ± 1.5*
$CD4^+$ cells	0.95 ± 0.50	3.1 ± 1.3*	2.4 ± 0.44*	2.8 ± 0.46*
$CD8^+$ cells	0.90 ± 0.49	2.0 ± 1.3	1.2 ± 0.46	3.1 ± 2.7*
Bone marrow	14 ± 3.2	15 ± 1.1	14 ± 2.2	15 ± 2.9
Thymus	2.6 ± 2.2	32 ± 9.3*	41 ± 25*	46 ± 29*

Data shown are the absolute numbers of cells ($\times 10^6$) in the indicated tissues (mean ± SD) in morphine-treated, morphine + naltrexone-treated, naltrexone-treated and placebo-treated mice.
*$P < 0.05$, significantly different from the morphine-treated group.

acetonitrile. The concentration of morphine was determined by liquid chromatography (LC)-mass spectroscopy (MS)/MS using a Waters ACQUITY ultra performance LC system, a Waters Quattro Premier XE triple quadrupole instrument with an ESI source (Waters, Milford, MA, USA), and MassLynx 4.1 software. Morphine was separated using an ACQUITY UPLC C18 column (1.7 µm, 100 mm × 2.1 mm inside diameter) equipped with an ACQUITY UPLC C18 guard column (Waters, Milford, MA, USA). The flow rate through the column at ambient temperature was 0.30 $mL \cdot min^{-1}$ with 80% acetonitrile and 20% H_2O containing 0.1% formic acid. The MS was operated in a positive mode with electrospray ionization. Source and desolvation temperatures were 120°C and 350°C respectively. Nitrogen was applied as the cone gas (10 $L \cdot h^{-1}$) and desolvation gas (700 $L \cdot h^{-1}$) and argon as the collision gas. Detection and quantification were performed using the multiple reactions monitoring mode with m/z 286/152 for morphine and 289/152 for d3-morphine.

Serum corticosterone concentrations

Corticosterone concentrations in sera were determined using the Corticosterone EIA Kit (Cayman, Ann Arbor, MI, USA). Briefly, 50 µL sera or standard, tracer and anti-corticosterone antiserum were added into the precoated plate. The plate was then incubated at room temperature with gentle shaking. After washing, the substrate reagent (Ellman's) was added into each well, incubated for 40 min, and analysed using Synergy HT Microplate Reader (BioTek, Winooski, VT, USA).

Fetal thymic organ culture

Fetal thymic organ culture (FTOC) was performed as described (Plum *et al.*, 1991; Ueno *et al.*, 2005). Briefly, thymic lobes were isolated from E15.5 fetus and placed on the surface of membrane filters (0.8 µm, Millipore, Billerica, MA, USA), which were supported by surgical gelfoam (Pfizer Inc, New York, NY, USA) soaked in Dulbecco's modified Eagle medium-10 media. Thymic lobes were cultured for 7 days, minced, and analysed by flow cytometry.

Statistics

All data are presented as mean ± SD. For day 7 analyses, 13 mice were implanted with morphine pellets and seven mice each were implanted with placebo, naltrexone, or morphine and naltrexone pellets. Data were analysed using a one-way ANOVA and a multiple comparisons *post hoc* analysis (Dunnett's method) between the morphine group and the control groups. For other time points, statistics were based on three to ten mice for each parameter. All data included represent at least three independent experiments and were analysed using two-tailed Student's *t*-tests. For the FTOC experiments, data were analysed using a one-way ANOVA and a multiple comparisons versus a control group *post hoc* analysis (Dunnett's method).

Results

Morphine induces the depletion of peripheral lymphocytes

Previous studies showed that morphine pellet implantation induces loss of thymic and splenic tissue weight and depletion of lymphocytes and then cells recover over time. We initiated our studies on day 7 after morphine pellet implantation, a time point at which the spleen has recovered most of its mass (Arora *et al.*, 1990) and after the thymocyte numbers reached their nadir (Sei *et al.*, 1991; Freier and Fuchs, 1993), and examined the lymphocyte populations. The total number of B-cells, $CD4^+$ T-cells and $CD8^+$ T-cells in the spleens and lymph nodes of morphine-treated animals remained significantly lower than in placebo-treated mice (Table 1).

Naltrexone treatment prevented the morphine-induced lymphocyte depletion, although variability within groups prevented some differences between the morphine and morphine plus naltrexone groups from being statistically different. It is feasible that the duration of morphine administration exceeded that of naltrexone in some mice, accounting for this variability. Few differences were noted between mice treated with placebo, naltrexone, or morphine plus naltrexone.

Morphine induces depletion of immature B-cells

To determine which subsets of splenic B-cells were most susceptible to morphine treatment, we analysed IgM and IgD expression on $B220^+$ cells, which allowed us to distinguish among FO B-cells (IgD^+IgM^{lo}), T2 B-cells (IgD^+IgM^+) and T1/MZ B-cells (IgM^+IgD^-). The percentages of $B220^+$ splenocytes that were in the T1/MZ B-cell populations were dramatically decreased in morphine-treated mice, as compared with the other groups (Figure 1A). In morphine-treated mice, 1.5 ± 0.5% of the splenic B-cells were T1 or MZ B-cells. Using CD21 to differentiate between T1 ($CD21^-CD23^-$) and MZ ($CD21^+CD23^-$) B-cells revealed that nearly all of the IgM^+IgD^- cells in morphine-treated mice were MZ B-cells in morphine-treated mice (Figure 1B). By contrast, 4.4 ± 0.6% of splenic B-cells in placebo-treated mice were T1 or MZ B-cells ($P < 0.001$) and 2.4 ± 0.4% of B-cells were MZ B-cells ($P = 0.015$). To further demonstrate that the IgM^+IgD^- cells lacked CD23 expression and were indeed T1 or MZ B-cells, we analysed CD23 expression on the IgM^+IgD^-, IgM^+IgD^+ and $IgM^{lo}IgD^+$ populations (Figure 1C). As previously reported (Loder *et al.*, 1999), T2 and FO B-cells expressed higher levels of CD23 than T1 and MZ B-cells, supporting our conclusion that the IgM^+IgD^- gate contained only T1 and MZ B-cells. These data indicated that the T1 B-cells were most sensitive to morphine treatment and suggested that B-cell development could be impaired by morphine treatment. In addition, the MZ B-cells were more sensitive to morphine treatment than T2 and FO B-cells, as the percentage of B-cells that were MZ B-cells declined after morphine treatment.

We traced B-cell development to the bone marrow and analysed the B-cell precursor populations. The absolute numbers of bone marrow cells in each group were nearly identical across treatment groups (Table 1). Further, the percentage of bone marrow cells expressing CD34 was similar across groups (Figure 1D). These data indicated that morphine did not induce the depletion of all hematopoietic precursors. In contrast to the $CD34^+$ population, the percentage of bone marrow cells that expressed B220 was reduced in morphine-treated mice (Figure 1E). In particular, pro-B/pre-B ($B220^+IgM^-$) cells and immature B ($B220^{lo}IgM^+$) cells were markedly decreased in morphine-treated mice, as compared with the control groups. In morphine-treated mice, 0.74 ± 0.35% of the bone marrow cells were pro-B/pre-B cells while 5.0 ± 2.4% of the bone marrow cells in placebo-treated mice were pro-B/pre-B-cells ($P < 0.001$). These data indicated that morphine treatment in mice impairs B-cell development by inducing the deletion of B-cell precursors.

B-cells recover from morphine-induced depletion via proliferation of B-cell precursors

By day 21 of the experiment, the number of B-cells in the spleen recovered to levels that were nearly identical to that of placebo-treated mice (Figure 2A). Because there were few differences between the three groups of control mice (placebo, naltrexone, and morphine plus naltrexone) at day 7, we used the placebo-treated mice as controls for the latter time points. We also compared the data throughout the experiment with a control group of untreated mice. We tested whether peripheral B-cells might proliferate as a mechanism by which splenic B-cells recover in number. Less than 2% of splenic B-cells were in the S, G2 or M phase of the cell cycle in any of the groups (Figure 2B), indicating that B-cell recovery did not occur via proliferation of the remaining cells.

Like splenic B-cells, the B-cell precursors in the bone marrow also recovered during the course of the experiment (Figure 2C). The percentage of bone marrow cells that were $B220^+$ cells were decreased in all groups 7 days after pellet implantation, but the morphine-treated mice had the largest decrease. By day 14, the percentage of bone marrow cells that were pro-B/pre-B cells in morphine-treated mice, placebo-treated mice and untreated mice were comparable. The immature B-cells and mature B-cells recovered more slowly in the morphine-treated mice than placebo-treated mice.

A possible mechanism by which bone marrow B-cell precursors could recover in number is through increased proliferation. The most dramatic increase in the percentage of cells in the cell cycle were found in the immature B-cell subset at day 14 (Figure 2D); 28 ± 11% of immature B-cells in morphine-treated mice were in the S, G2 or M phase of the cell cycle, as compared with 7.1 ± 3.1% of immature B-cells in placebo-treated mice ($P < 0.001$). In addition, more mature B-cells in the bone marrow were in the S, G2 or M phase in morphine-treated mice than placebo-treated mice at day 21.

Collectively, these data suggest that the mechanism by which the B-cells recover is primarily through increased proliferation of B-cell precursor populations. While splenic B-cells did not display elevated percentages of cells in the S, G2 or M phase of the cell cycle, $B220^{hi}$ bone marrow B-cells in morphine-treated mice did increase their proliferation. This suggests that different populations of B-cells may recover from depletion using different mechanisms.

Morphine treatment induces depletion of naïve and memory T-cell subsets

Morphine also induced the depletion of $CD4^+$ and $CD8^+$ T-cells in the spleens and lymph nodes (Table 1). To determine whether some T-cell subsets were more susceptible to the effects of morphine than others, we analysed whether naïve, central memory or effector memory subsets were preferentially depleted by morphine treatment. There were no differences across groups of mice between the percentages of $CD4^+$ or $CD8^+$ T-cells that were naïve ($CD44^{lo}CD62L^{hi}$), central memory ($CD44^{hi}CD62L^{hi}$) or effector memory ($CD44^{hi}CD62L^{lo}$) in the spleen (Figure 3) or in the lymph node (data not shown), indicating that all the major peripheral T-cell subsets were depleted by morphine to the same extent.

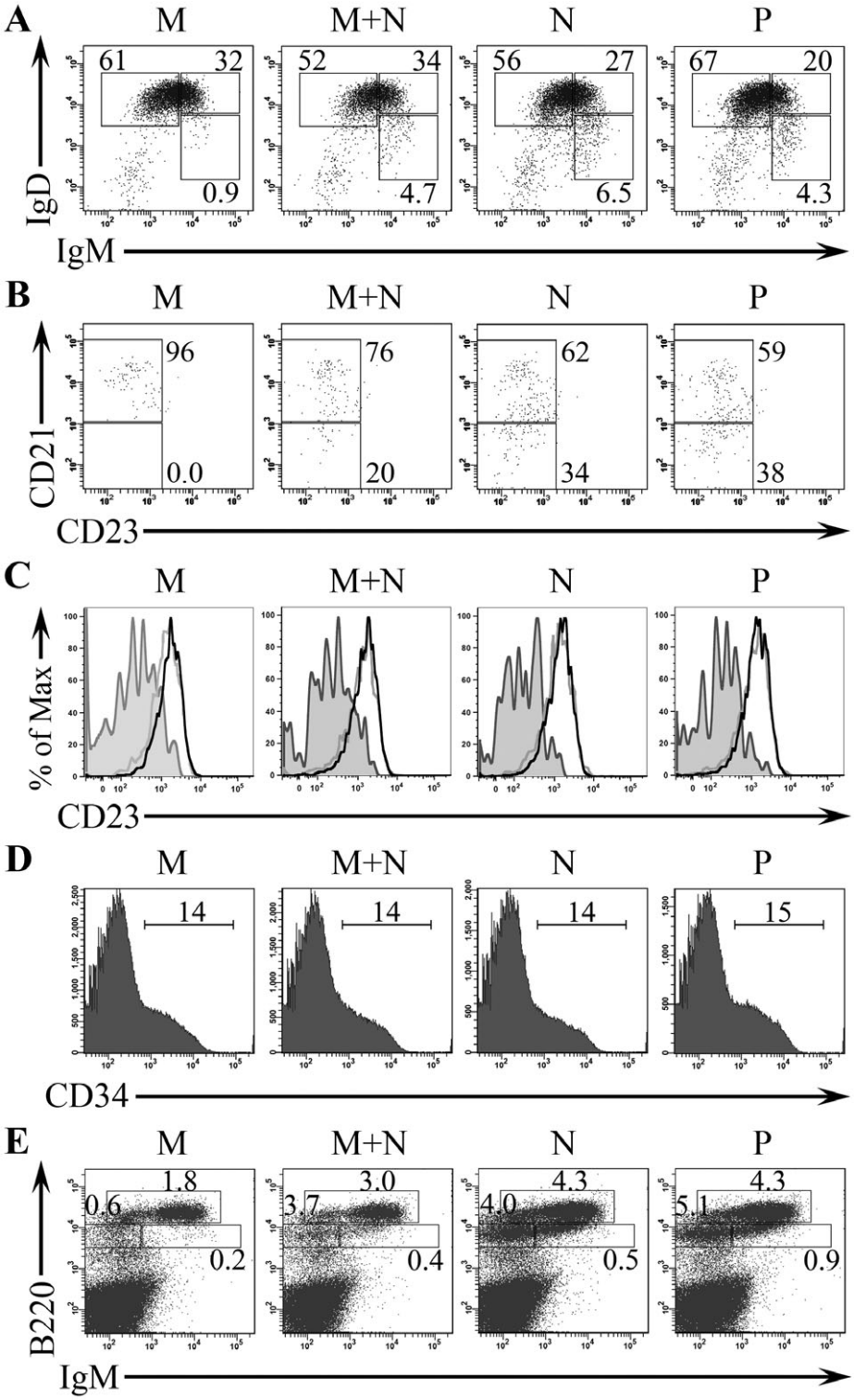


Figure 1

Morphine treatment depletes immature B-cells. Mice were treated with morphine (M), morphine and naltrexone (M + N), naltrexone (N) or placebo (P) and analysed 7 days later. (A) Splenocytes were gated on $B220^+$ cells and analysed for IgM and IgD expression. The percentages of B-cells in each gate are shown. (B) $B220^+IgM^+IgD^-$ cells from (A) were analysed for CD21 and CD23 expression. The percentages of gated cells that were T1 ($CD21^-$) and MZ ($CD21^+$) B-cells are shown. (C) $B220^+IgM^+IgD^-$ cells (*shaded histogram*), $B220^+IgM^+IgD^+$ cells (*grey line*), and $B220^+IgM^{lo}IgD^+$ cells (*black line*) from (A) were analysed for CD23 expression. (D) The percentages of bone marrow cells that expressed CD34 are shown. (E) Bone marrow cells were analysed for B220 and IgM expression. The percentages of cells in each gate are shown. Data shown represent one mouse from each group.

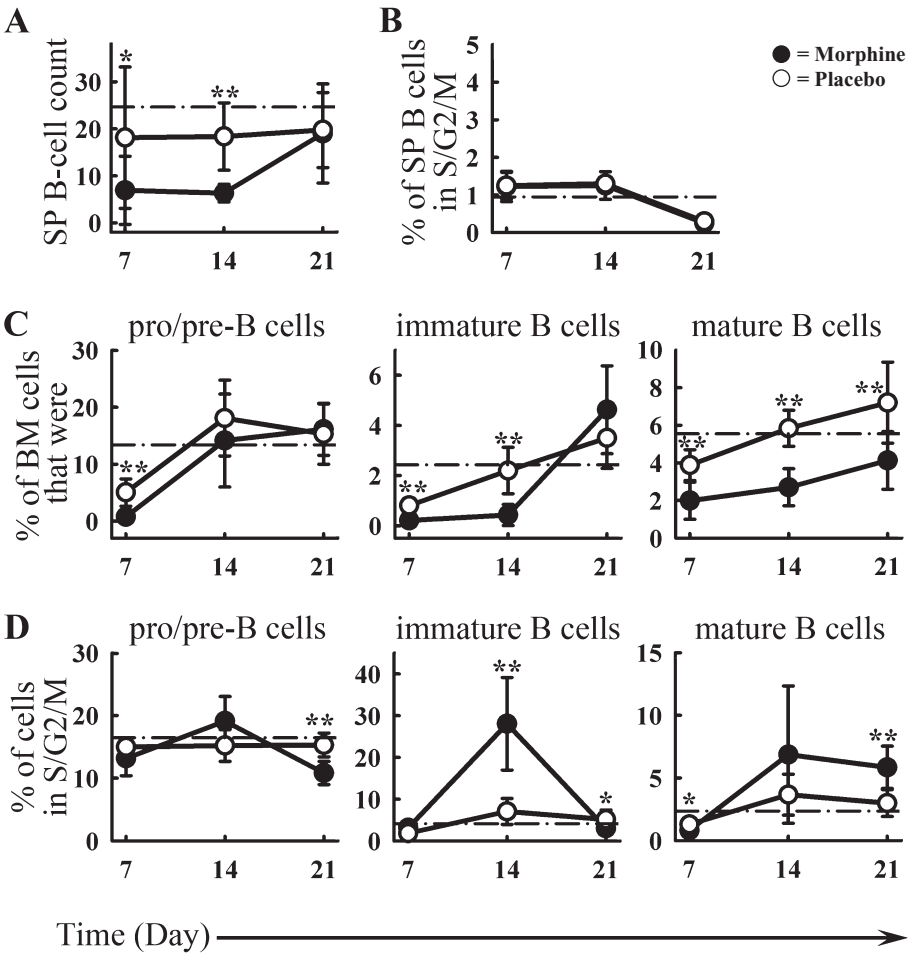


Figure 2

Recovery of B-cells after morphine treatment is due to proliferation of B-cell precursors. Mice were treated with morphine or placebo for 7, 14 or 21 days. Untreated control mice are shown as a dashed line. (A) The absolute numbers of splenic B-cells are shown. (B) The percentage of splenic B-cells in the S, G2 or M phase of the cell cycle are shown. (C) Bone marrow cells were analysed for B220 and IgM expression and the percentages of cells in each gate are shown. (D) Cells were gated as in (C) and analysed for DNA content. The percentages of cells in each gate that were in the S, G2 or M phase of the cell cycle are shown. *$P < 0.05$, **$P < 0.01$, comparing morphine-treated mice and placebo-treated mice.

Morphine treatment induces depletion of thymocytes undergoing selection

As shown in Table 1, the number of thymocytes per mouse was greatly reduced in morphine-treated mice, as compared with other groups. However, not all thymocyte subsets were depleted to a comparable extent. The population of cells most dramatically depleted by morphine treatment was the DP subset (Figure 4A), consistent with previous reports (Sei *et al.*, 1991; Freier and Fuchs, 1993). Within the DN population, DN3L and DN4 cells were most susceptible to depletion by morphine treatment (Figure 4B).

Consistent with our previous report in untreated mice (Zeng *et al.*, 2007), more than 80% of DN3L and DN4 thymocytes in control mice expressed TCRβ (Figure 4C) and many of these cells were in the S, G2 or M phase of the cell cycle (Figure 4D). Following morphine-treatment, the percentage of DN3L and DN4 thymocytes that expressed TCRβ was reduced, as compared with control groups. In morphine-treated mice, 31 ± 18% of DN3L cells expressed TCRβ and 40 ± 27% of DN4 cells expressed TCRβ ($P \leq 0.001$, as compared with placebo-treated mice). In addition, fewer $TCR\beta^+$ DN3E, $TCR\beta^+$ DN3L and $TCR\beta^+$ DN4 thymocytes were in the S, G2 or M phase of the cell cycle in morphine-treated mice than placebo-treated mice (Figure 4D), although only in the DN4 population did this difference reach statistical significance. These data indicate that morphine treatment blocks cell cycle progression and induces the depletion of $TCR\beta^+$ DN thymocytes.

The stage of T-cell development following the DN4 stage is the ISP $CD8^+$ thymocyte stage, defined as $CD8^+CD4^-CD24^{hi}TCR^{lo}$. ISP $CD8^+$ thymocytes were nearly absent in morphine-treated mice, whereas they represent approximately half of SP $CD8^+$ thymocytes in control mice (Figure 5A). Following the ISP $CD8^+$ stage, cells become DP thymocytes and then down-regulate CD8 to become TSP $CD4^+$ thymocytes, a population defined as $CD4^+CD8^{-/lo}CD24^{hi}$. Like ISP $CD8^+$ and DP thymocytes, TSP $CD4^+$ thymocytes were also nearly completely depleted in

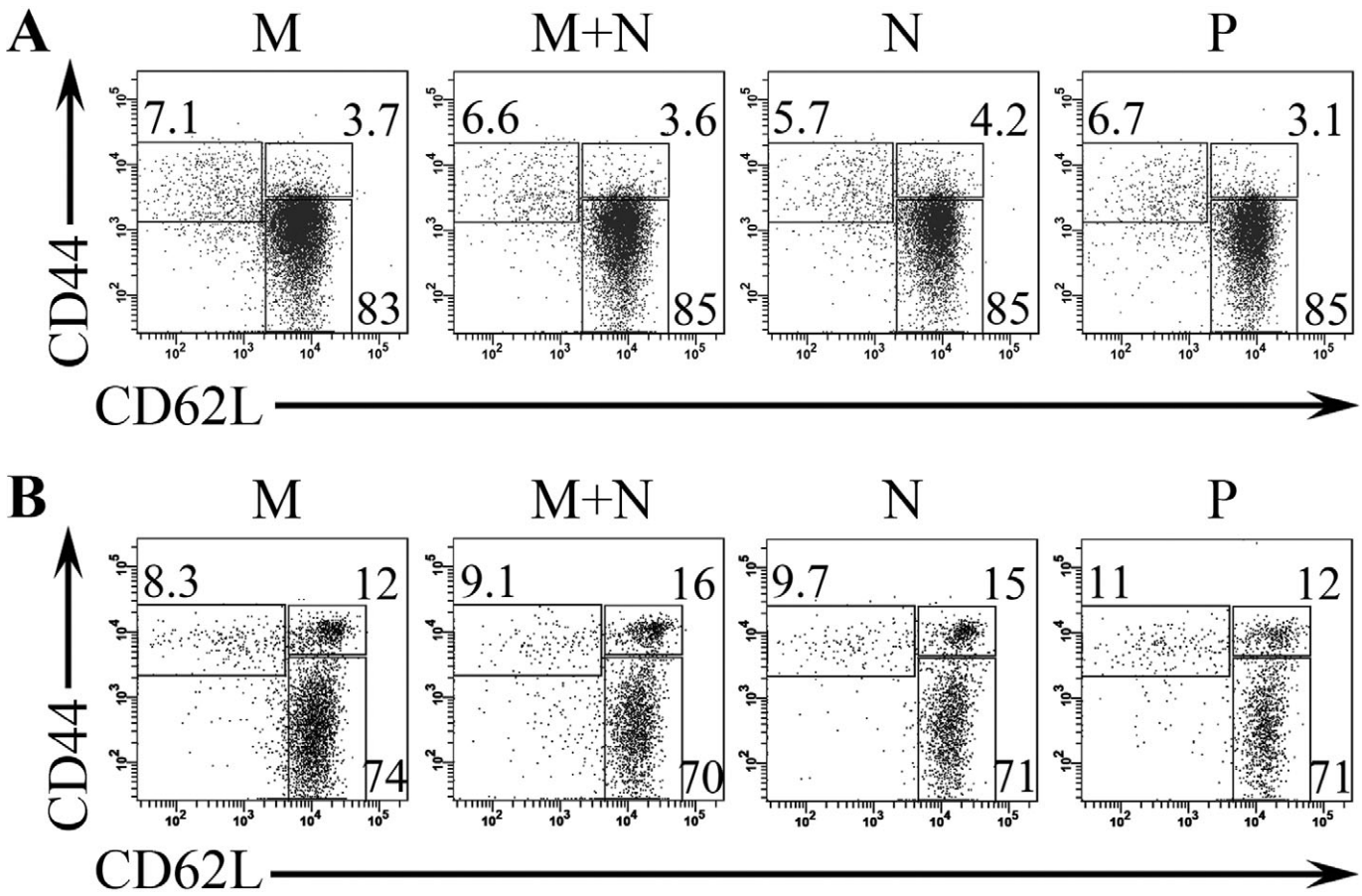


Figure 3

Morphine treatment does not affect the percentages of T-cells that are naïve, central memory and effector memory cells. Mice were treated with morphine (M), morphine and naltrexone (M + N), naltrexone (N) or placebo (P). Splenocytes were labelled with anti-CD4, anti-CD8, anti-CD44 and anti-CD62L 7 days after pellet implantation. The percentages of $CD4^+$ cells (A) and $CD8^+$ (B) in each gate are shown. Data shown represent one mouse from each group.

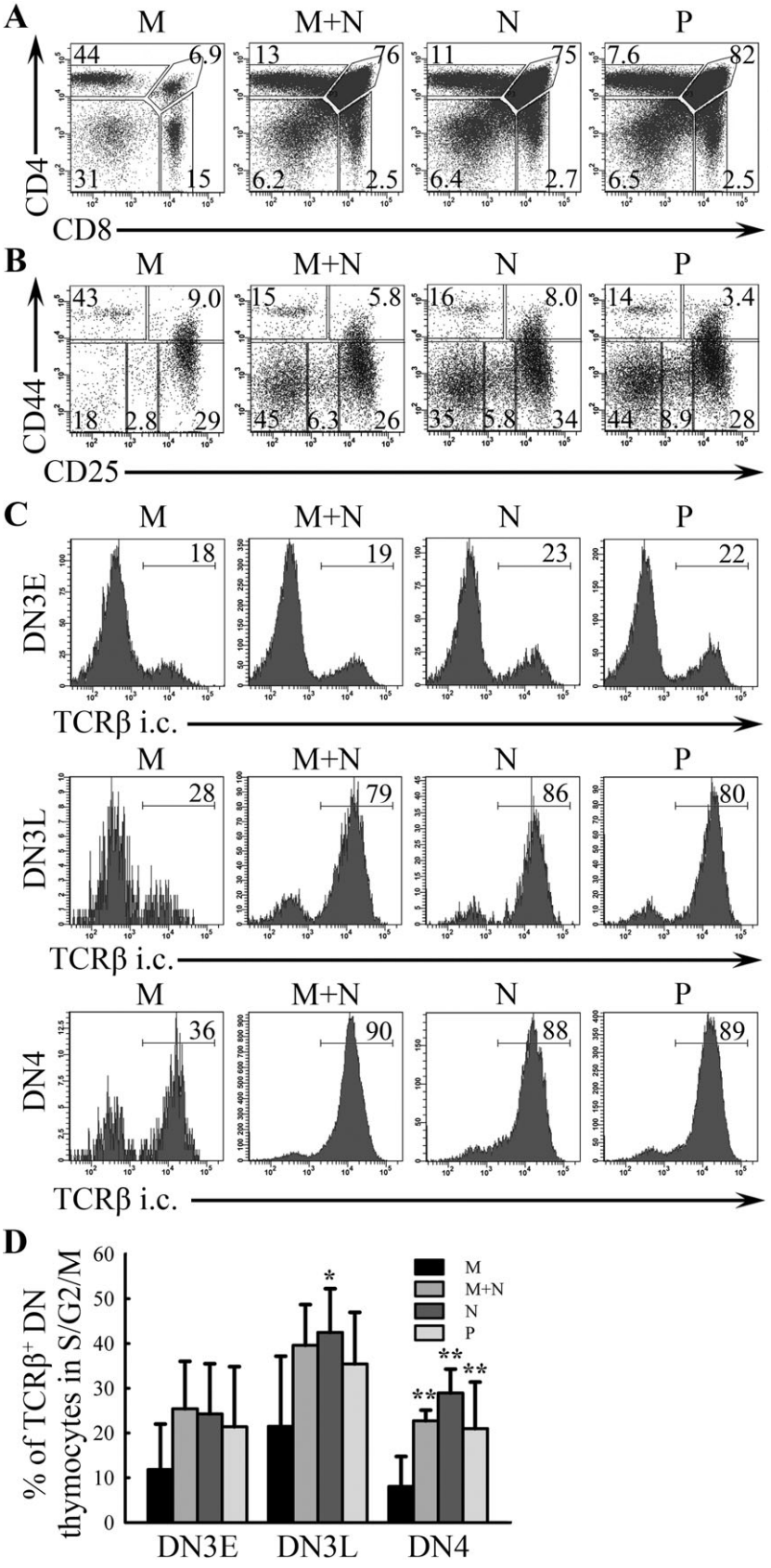
morphine-treated mice; nearly all SP $CD4^+$ thymocytes in morphine-treated mice were $CD24^{lo}$, indicating that the cells were mature (Figure 5B). These data suggest that cells in the developmental stages immediately following the β selection and positive selection steps are most susceptible to morphine-induced depletion.

Peripheral T-cell subsets recover via homeostatic proliferation

Unlike splenic B-cells, peripheral T-cells failed to completely restore their numbers to that of placebo-treated mice over the course of the experiment (Figure 6A). Although the amount of recovery in the peripheral T-cells was subtle and not statistically significant within the time frame of the experiment, we examined the peripheral T-cells for signs of homeostatic proliferation, the process by which lymphocytes expand in number in response to a lymphopenic environment. Seven days after pellet implantation, more $CD4^+$ and $CD8^+$ T-cells in the lymph node were in the S, G2 or M phase of the cell cycle than in placebo-treated mice ($P < 0.10$) (Figure 6B). In the spleen, more $CD4^+$ T-cells were in the S, G2 or M phase of the cell cycle 14 days after morphine treatment than placebo treatment ($P < 0.10$) (Figure 6B).

As a further test of whether T-cells underwent homeostatic proliferation, we examined whether the T-cells converted to a memory-like phenotype during the study (Figure 6C). T-cells undergoing homeostatic proliferation convert to a memory-like phenotype (Cho *et al.*, 2000; Clarke and Rudensky, 2000; Goldrath *et al.*, 2000; Murali-Krishna and Ahmed, 2000; Tanchot *et al.*, 2001). Greater percentages of $CD4^+$ T-cells in the spleens and lymph nodes on days 14 and 21 of the experiment displayed central memory and effector memory phenotypes in morphine-treated mice than placebo-treated mice. Likewise, more $CD8^+$ T-cells in morphine-treated mice had a central memory phenotype than in placebo-treated mice.

In summary, the analysis of peripheral T-cells suggested that the T-cells in morphine-treated mice underwent homeostatic proliferation. However, homeostatic proliferation is likely to be only a minor component of the recovery of T-cells following morphine treatment, as the T-cells failed to recover to normal numbers during the experiment.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8.0
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3.4
44
8.9
28
CD44
CD25
C
M
M+N
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P
18
19
23
22
DN3E
TCRβ i.c.
28
79
86
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DN3L
TCRβ i.c.
36
90
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DN4
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% of TCRβ+ DN thymocytes in S/G2/M
60
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40
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M
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DN3E
DN3L
DN4

Figure 4

Morphine treatment depletes thymocytes undergoing selection. Mice were treated with morphine (M), morphine and naltrexone (M + N), naltrexone (N) or placebo (P) and analysed 7 days later. Thymocytes were analysed for surface expression of CD4, CD8, CD44 and CD25 and intracellular expression of TCRβ. (A–C) Data shown represent one mouse from each group. (A) The percentages of thymocytes that were DN, DP, SP CD4$^+$ and SP CD8$^+$ cells are shown. (B) Thymocytes were gated on the DN population and analysed for CD44 and CD25 expression. The percentages of DN cells that were DN1, DN2, DN3E, DN3L and DN4 are shown. (C) Thymocytes were gated on DN3E, DN3L and DN4 thymocytes and analysed for intracellular TCRβ expression. The percentages of DN3E, DN3L and DN4 thymocytes that expressed TCRβ are shown. (D) TCRβ$^+$ DN3E, TCRβ$^+$ DN3L and TCRβ$^+$ DN4 thymocytes were analysed for their cell cycle status. The percentages of cells that were in the S, G2 or M phase of the cell cycle are shown. *$P < 0.05$, **$P < 0.01$, significantly different from morphine-treated mice.

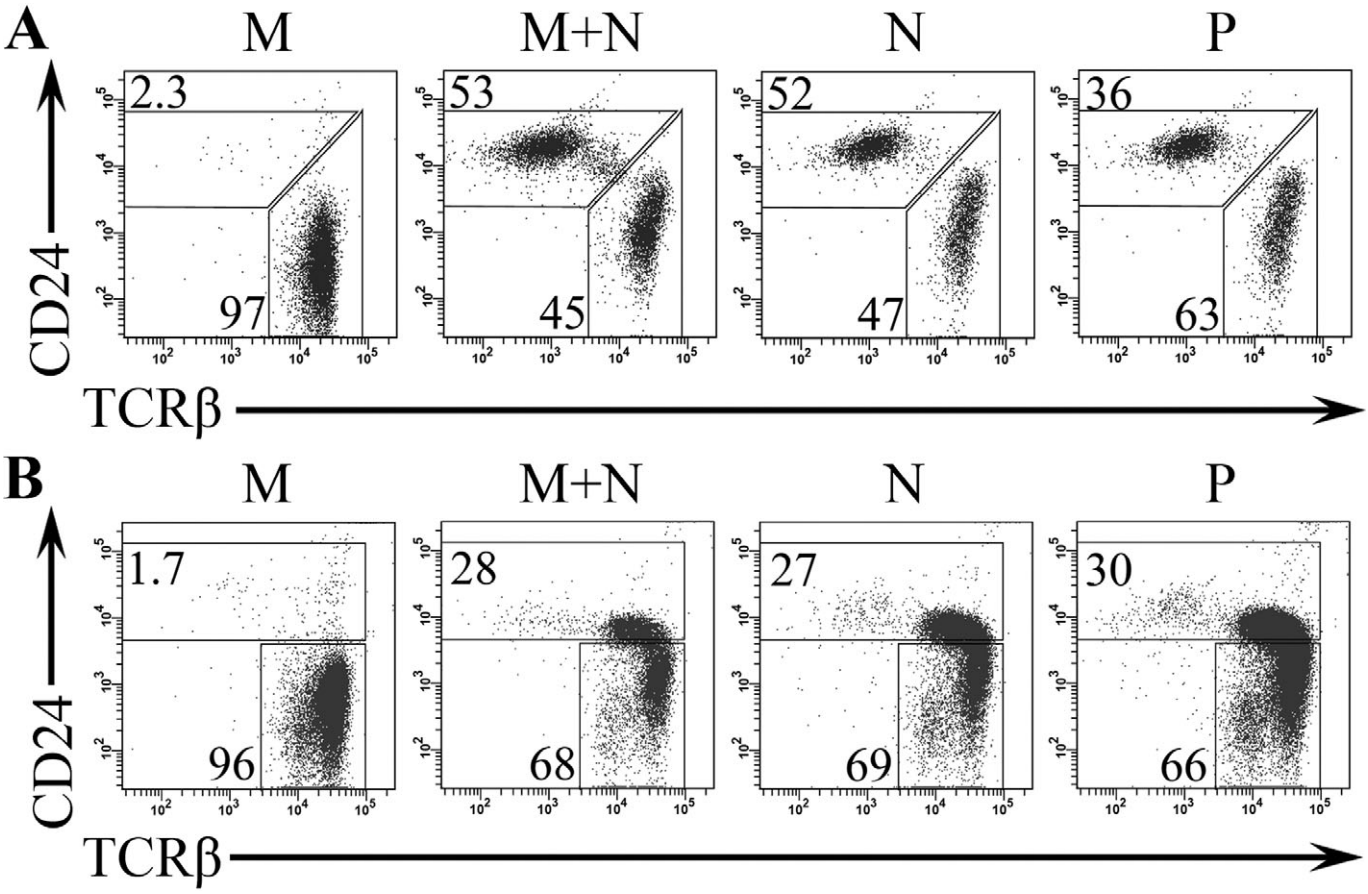

Figure 5

Morphine treatment induces depletion of ISP CD8$^+$ and TSP CD4$^+$ thymocytes. Mice were treated with morphine (M), morphine and naltrexone (M + N), naltrexone (N) or placebo (P) and analysed 7 days later. Thymocytes were surface labelled with anti-CD4, anti-CD8, anti-CD24 and anti-TCRβ. Cells were gated on SP CD8$^+$ (A) or SP CD4$^+$ (B) thymocytes and analysed for CD24 and TCRβ expression. The percentages of SP CD8$^+$ or SP CD4$^+$ thymocytes in each gate are shown. Data shown represent one mouse from each group.

T cells recover from morphine via increased proliferation of precursor cells

In the weeks following morphine pellet implantation, the number of thymocytes returned to nearly normal numbers (Figure 7A). In addition, the distribution of thymocytes into the DN, DP, SP CD4$^+$ and SP CD8$^+$ populations was fully restored within 3 weeks of morphine pellet implantation (Figure 7B). After 2 weeks of morphine treatment, the percentage of thymocytes that were DP slightly exceeded that of placebo-treated mice. The percentages of DN thymocytes that were DN1, DN2, DN3E, DN3L and DN4 returned to nearly normal levels within 3 weeks (Figure 7C). Also, the percentage of SP CD8$^+$ thymocytes that were ISP CD8$^+$ cells and the percentage of SP CD4$^+$ thymocytes that were TSP CD4$^+$ cells returned to normal levels (Figure 7D).

Because we observed a decrease in the percentage of DN3L and DN4 thymocytes that expressed TCRβ after 7 days of morphine treatment (Figure 4C), we examined these populations over time. The percentage of TCRβ$^+$ DN3E thymocytes in mice treated with morphine for 14 days exceeded that of placebo-treated mice (Figure 7E), suggesting that increased production of TCRβ$^+$ DN3E thymocytes could contribute to

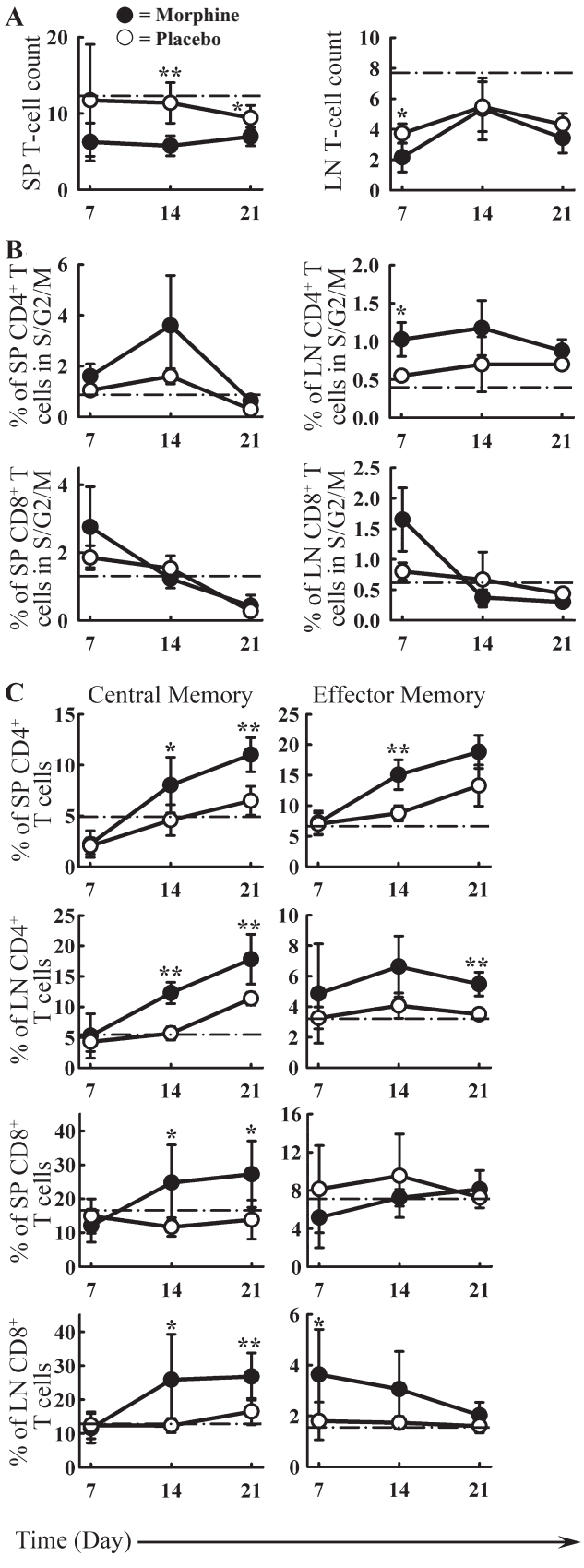

Figure 6

Peripheral T-cells recover from morphine-induced depletion via homeostatic proliferation. Mice were treated with morphine or placebo for 7, 14 or 21 days. Untreated control mice are shown as a dashed line. (A) The absolute number of splenic (SP) and lymph node (LN) T-cells are shown. (B) The percentages of SP and LN $CD4^+$ and $CD8^+$ cells that were in the S, G2 or M phase of the cell cycle are shown. (C) Spleen and lymph node cells were analysed as described in the legend to Figure 3. The percentages of cells that were central memory or effector memory are shown. $^*P < 0.05$, $^{**}P < 0.01$.

the restoration of the T-cell populations. We next examined mechanisms by which this rebound in the $TCR\beta^+$ DN3E population could occur. We found that a greater percentage of DN1, DN2 and $TCR\beta^+$ DN3E thymocytes were in the S, G2 or M phase of the cell cycle in morphine-treated mice than placebo-treated mice (Figure 7F). The percentages of $TCR\beta^+$ DN3L and $TCR\beta^+$ DN4 thymocytes in the S, G2 or M phase of the cell cycle did not differ between the groups of mice on days 14 and 21 of the experiment (data not shown). However, more DP thymocytes were in the S, G2 or M phase of the cell cycle 7 days after pellet implantation in morphine-treated mice than placebo-treated mice (Figure 7F).

In summary, these data suggest that thymocyte populations recover following morphine treatment via increased proliferation of DN1, DN2, $TCR\beta^+$ DN3E and DP thymocytes. This proliferation seen in morphine-treated mice may cause the percentage of thymocytes that were $TCR\beta^+$ DN3L, ISP $CD8^+$ and DP cells to exceed transiently that of placebo-treated mice.

Morphine pellet implantation results in sustained plasma morphine levels and a transient increase in corticosterone levels

Previous reports suggested that morphine treatment in mice could induce elevated corticosteroid production (Briggs and Munson, 1955; George and Way, 1955; Bryant *et al.*, 1991; Sei *et al.*, 1991; Hernandez *et al.*, 1993). To test whether this occurred during our experiments, we examined serum morphine and corticosterone levels in mice from each group. Serum concentrations of free, unconjugated morphine reached an average of 4.3 ± 0.56 μM within 6 h of implantation, decayed to 1.3 ± 0.61 μM within 7 days, and was 0.22 ± 0.11 μM on day 21 (Figure 8A). Within 6 h of morphine pellet implantation, corticosterone levels reached levels that were 5.3 ± 1.6-fold greater than pre-implantation levels (Figure 8B). Mice receiving placebo, naltrexone, and morphine plus naltrexone had corticosterone levels 6 h after implantation that were approximately twofold greater than presurgery levels.

These data indicated that lymphocyte populations were depleted while corticosterone levels were elevated and recovered when corticosterone levels returned to nearly baseline levels. In addition, lymphocyte recovery occurred while morphine levels remained at physiologically significant levels.

Morphine does not directly impair T-cell development

We tested whether morphine could directly impair T-cell development using FTOC. Thymi were collected from day

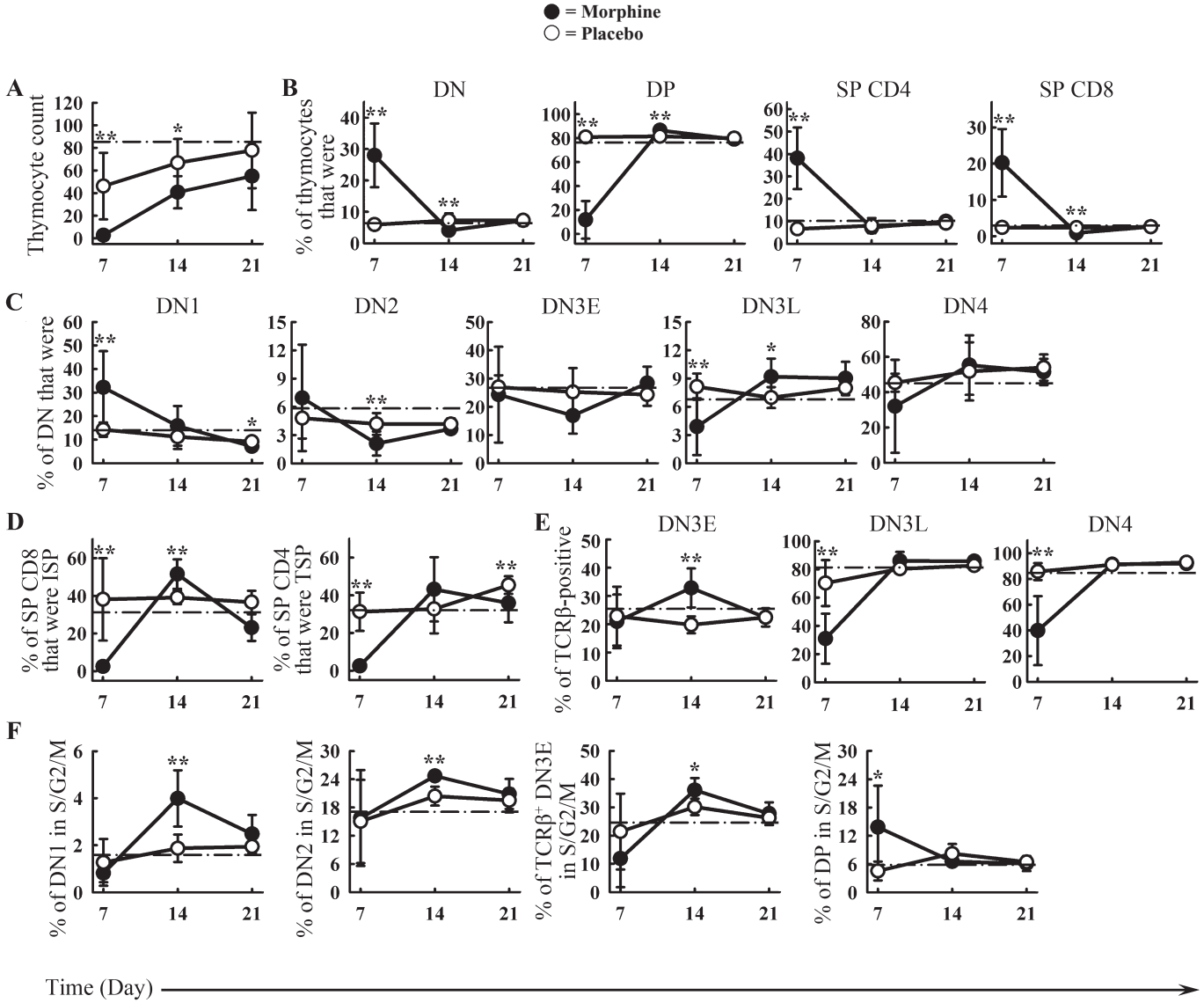

Figure 7

Recovery of T-cells via increased proliferation of precursor cells. Mice were treated with morphine or placebo for 7, 14 or 21 days. Untreated control mice are shown as a dashed line. Thymocytes were analysed as described in Figures 4 and 5. (A) The absolute number of thymocytes is shown. (B) The percentages of thymocytes that were DN, DP, SP CD4$^+$ and SP CD8$^+$ thymocytes and shown. (C) The percentages of DN thymocytes that were DN1, DN2, DN3E, DN3L and DN4 thymocytes are shown. (D) The percentages of SP CD8$^+$ and SP CD4$^+$ thymocytes that were ISP CD8$^+$ and TSP CD4$^+$ thymocytes, respectively, are shown. (E) The percentages of DN3E, DN3L and DN4 thymocytes that expressed TCRβ are shown. (F) The percentages of DN1, DN2, TCRβ$^+$ DN3E and DP thymocytes that were in the S, G2 or M phase of the cell cycle are shown. *P < 0.05, **P < 0.01.

E15.5 fetuses and cultured in the presence of morphine, morphine plus naltrexone, or no drug. The higher dose of morphine used in this experiment was based on the peak concentration of morphine in sera of morphine-treated mice. The lower dose of morphine used resembled the concentration of morphine in sera of mice treated with morphine for 7 days. In addition, dexamethasone was added to some samples to mimic the effects of increased corticosteroid production *in vivo*. The concentration of dexamethasone used in this assay was approximately one-tenth the concentration of corticosterone observed *in vivo*. Because dexamethasone is approximately 10-fold more potent at inducing apoptosis of thymocytes than corticosterone (Cohen and Duke, 1984), the relative activity of dexamethasone in our assay is comparable to the activity of corticosterone seen *in vivo*.

Similar numbers of thymocytes were recovered from thymi cultured with morphine, morphine plus naltrexone, and no drug (Figure 9A). Further, the distributions of DN, DP, SP CD4$^+$ and SP CD8$^+$ thymocytes were similar between morphine-treated samples and the control samples (Figure 9B). To ensure that morphine was not rapidly degraded in culture, we analysed the concentration of mor-

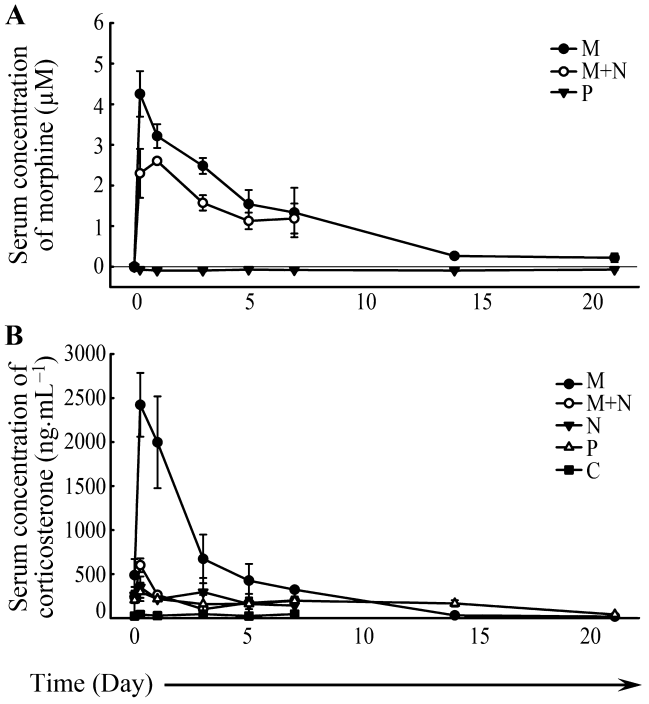

Figure 8

Morphine pellet implantation leads to sustained serum morphine levels and a transient increase in serum corticosterone levels. Mice were implanted with pellets containing morphine (M), morphine and naltrexone (M + N), naltrexone (N), or placebo (P) and blood was collected at the indicated time points. The concentrations of morphine (A) and corticosterone (B) in sera of mice treated for the indicated lengths of time are shown. Untreated control mice (C) were also analysed (B).

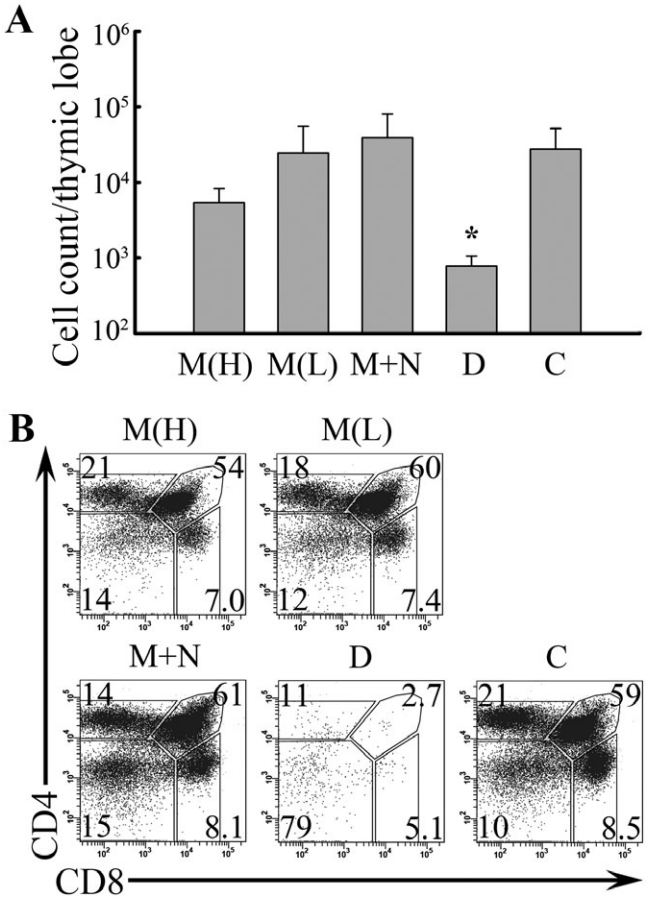

Figure 9

Morphine does not directly impair thymic development. FTOC was performed as described in *Methods* in the presence of 6 μM [M(H)] or 1 μM [M(L)] morphine, 6 μM morphine plus 6 μM naltrexone (M + N), 1 μM dexamethasone (D), or media (C). (A) The absolute numbers of thymocytes recovered after 7 days of culture are shown. (B) Thymocytes were analysed for CD4 and CD8 expression. The percentages of cells that were DN, DP, SP CD4$^+$ and SP CD8$^+$ thymocytes are shown. Dot plots represent one mouse from each group.

phine in a separate tissue culture experiment and found that the concentration remained stable for at least 10 days (data not shown).

In contrast to morphine-treated samples, few thymocytes were harvested from dexamethasone-treated samples and most thymocytes recovered were DN thymocytes (Figure 9). These data suggested that morphine does not directly induce loss of thymocyte populations. Rather, the depletion of thymocytes observed *in vivo* was most likely to be due to the surge in corticosterone levels.

Discussion

In this report, we provide a detailed analysis of B- and T-cell populations that remain after morphine treatment in mice. This level of description directed us toward discovering the mechanisms by which lymphocytes recover following morphine-induced depletion. We found that the mechanisms of recovery included a combination of accelerated B- and T-cell development and homeostatic proliferation of peripheral T-cells. Understanding these mechanisms will enable us to comprehend the immunological changes observed in patients using or abusing opioids.

The populations of lymphocyte precursors that were most susceptible to morphine-induced depletion were generally the populations that normally undergo positive selection. For example, the pro-B/pre-B-cell, immature B-cell, and T1 B-cell subsets were dramatically depleted in morphine-treated mice (Figure 1). The pre-B-cell and immature B-cell subsets are the populations in which rearrangements of the immunoglobulin genetic loci occur and the cells are under selection pressure to respond to pre-B cell receptor (BCR) and BCR signals. The T1 stage of B-cell development is a transient stage that is also subjected to selection pressure (Chung *et al.*, 2003). T1 cells also undergo a high degree of turnover (Yankee *et al.*, 2005). Although it is not clear whether MZ B-cells continuously receive selection signals, MZ B-cells proliferate and are eliminated at a higher rate than T2 and FO B-cells (Yankee *et al.*, 2005). MZ B-cells are also susceptible to morphine-induced depletion (Figure 1), suggesting that cells that

undergo high rates of turnover are particularly sensitive to morphine-induced depletion.

T-cell precursors undergoing selection were also depleted following morphine treatment. Namely, TCRβ$^+$ DN thymocytes, ISP and DP thymocyte subsets include cells that recently completed genomic rearrangements at the TCRβ and TCRα loci and receive signals from the pre-TCR or TCR to undergo positive selection. These populations were also highly sensitive to morphine-induced depletion (Figure 4). The TSP CD4$^+$ thymocyte population, which was depleted in morphine-treated mice, consists primarily of cells that completed positive selection (Dalheimer *et al.*, 2009) and receive signals that determine whether the cells become mature SP CD4$^+$ or mature SP CD8$^+$ cells (Lundberg *et al.*, 1995; Suzuki *et al.*, 1995; Lucas and Germain, 1996). In our studies, we could not determine whether TSP CD4$^+$ thymocytes were direct targets of the effects of morphine or whether this population was depleted because of the dramatic losses in the cell populations that precede the TSP CD4$^+$ stage.

The stages of early T-cell development that were most resistant to morphine treatment were the DN1 and DN2 stages (Figure 4A). These stages of T-cell development are unique in that they are highly dependent upon Notch signalling for their survival (Radtke *et al.*, 2004; Robey and Bluestone, 2004). After cells express TCRβ and cells proceed through the DN3L, DN4, ISP and DP stages, Notch1 expression declines and the cells no longer respond to Notch ligands (Huang *et al.*, 2003; Fiorini *et al.*, 2009). Thus, cells that respond to Notch ligands were relatively resistant to morphine-induced cell depletion. This observation is similar to previous studies that demonstrated that Notch signalling could render thymocytes resistant to corticosteroid-induced cell death (Deftos *et al.*, 1998), implying that corticosteroids, not morphine, are responsible for the thymocyte depletion observed in our studies. Indeed, we observed a large increase in serum corticosterone levels following morphine pellet implantation (Figure 8B), consistent with previous reports (Bryant *et al.*, 1988b; Roy *et al.*, 2001).

We tested whether morphine could directly impair T-cell development using FTOC. In this system, developing T-cells are provided as the stroma required for T-cell development *in vivo*. Cells are able to survive, proliferate and undergo positive and negative selection using FTOC. Further, because T-cell development is a lifelong process, the FTOC system likely mimics T-cell development at all stages of life. Morphine had no effect on the expansion and differentiation of thymocytes (Figure 9). By contrast, few thymocytes were recovered from dexamethasone-treated cultures. These observations were consistent with previous reports showing that morphine-induced thymic hypoplasia did not occur in adrenalectomized mice (Sei *et al.*, 1991). These data indicate that morphine does not directly impair T-cell development. Rather, the corticosterone production that resulted from morphine treatment was more likely to have prevented thymocyte expansion and differentiation.

The toxic effects of corticosteroids in the FTOC experiment are consistent with previous reports showing that corticosteroids induce apoptosis of developing B and T-cells. *In vivo* administration of corticosterone resulted in a dramatic loss of thymic weight (DePasquale-Jardieu and Fraker, 1980; Garvy *et al.*, 1993; Laakko and Fraker, 2002). Further, the immature T-cell populations were most susceptible to corticosteroid treatment (Khalid *et al.*, 1983; Gruber *et al.*, 1994; Ayala *et al.*, 1995; Tarcic *et al.*, 1998). Like developing T-cells, immature B-cells were also susceptible to corticosterone-induced apoptosis, while other bone marrow cell types were resistant to corticosteroids (Vines *et al.*, 1980; Ku and Witte, 1986; Sabbele *et al.*, 1987; Garvy *et al.*, 1993; Laakko and Fraker, 2002). The selective loss of lymphoid precursors in corticosterone-treated mice was similar to morphine-induced depletion of lymphocyte precursors.

Like immature lymphocytes, mature B and T-cells were also susceptible to the depleting effects of morphine (Table 1), consistent with previous reports (Arora *et al.*, 1990; LeVier *et al.*, 1994; Hilburger *et al.*, 1997). While depletion of immature lymphocyte was most likely due to the effects of corticosteroids, depletion of mature lymphocytes was more likely to be due to the interplay between corticosteroids and the μ-opioid receptor. Stress responses, which lead to elevated corticosteroid levels, can trigger lymphocyte apoptosis in a manner dependent on elevated CD95/Fas expression and expression of the μ-opioid receptor on splenocytes (Yin *et al.*, 1999; Yin *et al.*, 2000; Wang *et al.*, 2002). Consistent with a role of corticosteroids in the depletion of splenocytes, the total number of splenocytes in mice decreased within 2 days of systemic prednisolone treatment (Lundin and Jarplid, 1973; Bach *et al.*, 1975). *In vitro* experiments showed that prednisolone could induce apoptosis of mature lymphocytes (Batra *et al.*, 1966; Nieto and Lopez-Rivas, 1992; Perandones *et al.*, 1993). In our studies, we provided exogenous morphine and induced a corticosteroid response. Thus, cell depletion in our studies is likely due to the direct effects of morphine as well as the indirect effect of corticosteroids influencing endogenous opioid production.

We observed a slight increase in serum corticosterone levels in all mice undergoing pellet implantation, although the increase was significantly greater in morphine-treated mice than control mice (Figure 8B). This transient increase in corticosterone levels probably accounts for the fact that the placebo pellets also affected some parameters analysed within the B- and T-cell populations (Figures 2, 6 and 7).

The serum concentrations of morphine observed in our studies resembled physiologically relevant levels. While many opioid addicts have sustained serum morphine concentrations less that 0.1 μM (Aderjan *et al.*, 1995), chronic morphine use can result in levels greater than 4 μM (Rook *et al.*, 2006). This indicates that, while the levels of morphine observed in our studies are high, they are achievable in humans.

At least two mechanisms contributed to the recovery of peripheral B-cell and T-cells following morphine-induced depletion: increased lymphocyte production and homeostatic proliferation. For both B and T-cells, there was an increase in the production of progenitor populations. Immature B-cells and DN1, DN2, TCRβ$^+$ DN3E and DP thymocytes proliferated more extensively 2 weeks after morphine pellet implantation than in control mice (Figures 2D and 7F). This proliferation would create a larger pool of cells to be released from the primary lymphoid organs into the secondary lymphoid organs.

These data are consistent with experiments in which dexamethasone was injected into mice and elevated serum levels

of IL-7 and stromal-derived factor-1α were observed (Zubkova *et al.*, 2005). These cytokines are critical for B- and T-cell development (Peschon *et al.*, 1994; von Freeden-Jeffry *et al.*, 1995; Maki *et al.*, 1996). The primary function of IL-7 in T-cell development is to promote the survival and proliferation of DN1 and DN2 thymocytes. In B-cells, IL-7 promotes differentiation beyond the pre-B-cell stage. Thus, IL-7 levels are likely to be elevated in morphine-treated mice and could then induce the increased proliferation of lymphoid precursors seen in this study.

In secondary lymphoid organs, B-cells recovered to nearly normal levels in the 3 weeks after morphine pellet implantation while T-cell numbers lagged. During the recovery, we did not observe an increase in the percentage of B-cells that were in the S, G2 or M phase of the cell cycle (Figure 2B), suggesting that homeostatic proliferation was unlikely to be a mechanism by which peripheral B-cell numbers recovered. By contrast, peripheral T-cells displayed evidence of homeostatic proliferation, yet their numbers failed to recover to normal levels (Figure 6). The consequence of this homeostatic proliferation is that an increased percentage of T-cells had a memory phenotype and function. Memory T-cells can be activated by a lower concentration or affinity of antigen than naïve cells and memory cells do not have a strict requirement for co-stimulation. This increase in memory cells is similar to what is observed in elderly patients, who are at increased risk for autoimmune disease (Ramos-Casals *et al.*, 2003; Dorshkind *et al.*, 2009). This increase in memory cells also resembles that observed in patients undergoing therapy that causes lymphocyte depletion, such as cancer chemotherapy or anti-T-cell therapy. In these patients, the conversion to memory cells is associated with autoimmune disease and transplant rejection (Williams *et al.*, 2007; Askenasy *et al.*, 2010). Thus, it is possible that depletion and recovery of the T-cell population may render patients susceptible to autoimmune disease.

The data here demonstrate that administering morphine to mice causes dramatic T-cell depletion and the T-cells that repopulate the lymphoid tissues have different phenotypes from those expressed before morphine administration. Additional mechanisms by which morphine could impair the immune response have been proposed. For example, morphine has been reported to inhibit mitogen-induced proliferation, Th1/Th2 differentiation, antibody production and chemotaxis (Hernandez *et al.*, 1993; Flores *et al.*, 1994; Eisenstein *et al.*, 1995; Flores *et al.*, 1995; Grimm *et al.*, 1998; Wetzel *et al.*, 2000; Szabo *et al.*, 2003; Roy *et al.*, 2004).

In summary, we determined the mechanisms by which morphine administration to mice caused depletion of the B- and T-cell populations. We found that morphine had no direct effect on thymocyte populations and the profound depletion of thymocytes seen *in vivo* was most likely caused by an increase in the serum corticosterone levels. For mature lymphocyte subsets, depletion was most likely to be caused by a combination of corticosteroids and the direct effects of morphine. Once the corticosterone levels returned to control levels, the lymphocytes recovered even though morphine was still present at significant levels. This recovery was due to a combination of increased B- and T-cell production and homeostatic proliferation. Homeostatic proliferation was most evident among T-cells and not detected in B-cells, indicating that different populations of lymphocytes have different propensities to undergo homeostatic proliferation in morphine-treated mice.

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Conflict of interest

None.

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