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Transplantation with Lewis bone marrow induces the reinstatement of cocaine-seeking behavior in male F344 resistant rats

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ABSTRACT

One of the main challenges to understand drug addiction is defining the biological mechanisms that underlie individual differences in recidivism. Studies of these mechanisms have mainly focused on the brain, yet we demonstrate here a significant influence of the peripheral immune system on this phenomenon. Lewis (LEW) and Fischer 344 (F344) rats have different immunological profiles and they display a distinct vulnerability to the reinforcing effects of cocaine, with F344 more resistant to reinstate cocaine-seeking behavior. Bone marrow from male LEW and F344 rats was transferred to male F344 rats (F344/LEW-BM and F344/F344-BM, respectively), and these rats were trained to self-administer cocaine over 21 days. Following extinction, these animals received a sub-threshold primer dose of cocaine to evaluate reinstatement. F344/LEW-BM but not F344/F344-BM rats reinstated cocaine-seeking behavior, in conjunction with changes in their peripheral immune cell populations to a profile that corresponded to that of the LEW donors. After cocaine exposure, higher $CD4^+$ T-cells and lower $CD4^+CD25^+$ T-cells levels were observed in F344/LEW-BM rats referred to control, and the splenic expression of *Il-17a*, *Tgf-β*, *Tlr-2*, *Tlr-4* and *Il-1β* was altered in both groups. We propose that peripheral T-cells respond to cocaine, with $CD4^+$ T-cells in particular undergoing Th17 polarization and generating long-term memory, these cells releasing mediators that trigger central mechanisms to induce reinstatement after a second encounter. This immune response may explain the high rates of recidivism observed despite long periods of detoxification, shedding light on the mechanisms underlying the vulnerability and resilience of specific individuals, and opening new perspectives for personalized medicine in the treatment of relapse.

1. Introduction

Cocaine addiction is one of the most overwhelming examples of experience-dependent behavioral changes that may be permanent, involving lifelong memory. However, this phenomenon cannot be fully explained by the neural alterations reported following chronic exposure to this drug (Edwards and Koob, 2010; Everitt and Robbins, 2005; Hyman et al., 2006; Lüscher and Malenka, 2011; Robinson and Kolb, 2004; Thomas et al., 2009). The deleterious effects of psychostimulants are not exclusively behavior-related, as immune function is also compromised (Assis et al., 2008; Kubera et al., 2008; Lo Iacono et al., 2018). In fact, while the brain's reward system can influence the activity of the peripheral immune system, this may also be directly modulated by drugs of abuse, and cocaine may influence immune cells by provoking the release of immunotransmitters (e.g. dopamine) and immunomodulators (cytokines) that act in autocrine/paracrine loops (Araos et al., 2015; Assis et al., 2011; Ben-Shaanan et al., 2018; Bergquist et al., 1994; Cosentino et al., 2007; Nistico et al., 1994; Oberbeck, 2006; Pellegrino and Bayer, 1998; Yamada and Nabeshima, 2004). Moreover, as cocaine reaches leukocytes before crossing the blood–brain barrier (BBB), the

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effect of cocaine on immunocytes may even precede its effects in the central nervous system (CNS). The effects of cocaine on immunotransmission may also influence the CNS response to this drug since the immune system regulates learning, memory, neural plasticity and neurogenesis (Yirmiya and Goshen, 2011). Indeed, immunological dysfunction has been linked with neuropathologies, some of which can be reverted by bone marrow (BM) transplantation (Chen et al., 2010; Derecki et al., 2012; Díaz et al., 2019, 2015, 2012; Kwan et al., 2012; Leonard, 2010; Liu et al., 2019a; Miyaoka et al., 2017).

Due to differences in their vulnerability to drugs of abuse, the inbred Fischer 344 (F344) and Lewis (LEW) rat strains have been used to study the biological correlates of addiction (Cadoni, 2016; Haile et al., 2001; Kosten et al., 1997; Kosten and Ambrosio, 2002; Miguéns et al., 2011; Sanchez-Cardoso et al., 2007; Sánchez-Cardoso et al., 2009). The LEW strain is more sensitive than the F344 rats in terms of reinstating cocaine-seeking behavior, even after receiving sub-threshold cocaine priming (Kruzich and Xi, 2006; Miguéns et al., 2013). In addition, differences in the immune system of these rats have also been reported (Fecho et al., 2007; Macho et al., 2008; Mattapallil et al., 2008; Wilder et al., 2000), whereby F344 rats have fewer mononuclear cells than LEW rats, a lower proportion of $CD4^+$ T-cells and less intense CD4 staining (Griffin and Whitacre, 1991). We also found differences between the T/B-cell ratio, which in LEW rats is nearly double that of F344 rats (Ucha-Tortuero, 2012).

The behavioral differences between LEW and F344 rats, and those in their immune responses, make these strains an attractive model to study the effects of cocaine on the peripheral immune system and its relationship with behavior. We predicted that the differences in the lymphocyte populations, particularly the higher prevalence of $CD4^+$ T-cells in LEW rats, would influence drug-evoked long-term memory by triggering mechanisms of immune recognition, explaining the heightened sensitivity of these rats to relapse. Accordingly, to study the potential effects of the immune system on relapse, we transferred BM from LEW to irradiated F344 rats, assessing whether these LEW BM cells modify the cocaine-induced behavioral response of F344 rats in a paradigm of self-administration and reinstatement of drug-seeking behavior.

2. Methods

An abbreviated version of the methods employed is presented here. For further details, see the Supplementary material.

2.1. Animals

Naïve male F344 rats (n = 13) were randomly transplanted with BM from male F344 rats (F344/F344-BM, control, n = 7) or male LEW rats (F344/LEW-BM, experimental, n = 6), using F344 rats and transgenic green fluorescent protein (GFP^+) LEW rats as donors. Male rats were used in accordance to the previous data reported by Miguéns et al. (2013). All the rats were maintained in a temperature-controlled vivarium on a 12 h light/dark cycle, with food and water *ad libitum* unless otherwise specified. All efforts were made to minimize animal suffering and the number of animals used, in accordance with European (directive 2010/63/EU) and Spanish Legislation (Law 32/2007, RD 53/2013). All the protocols used in this study were approved by the local ethical committees (UNED and USAL).

2.2. BM transplantation

Ionizing radiation was used for efficient BM cell transplantation (Alvarez-Dolado et al., 2003; Massengale et al., 2005). On post-natal day 19 (P19), the BM of the recipients was ablated with a dose of 7.5 Gy (minimal lethal dose) radiation using a Gammacell 1000 Elite gamma irradiation device (MDS Nordion, Ottawa, Canada) with a ^{137}Cs source (Díaz et al., 2012, 2011; Recio et al., 2011). At P20, recipients were transplanted with BM from F344 or LEW-GFP^+ donors (see Fig. 1A). The donors (8- to 10-weeks-old) were sacrificed with CO_2, decapitated, and BM stem cells were isolated from their tibiae and femurs, as described previously (Díaz et al., 2012). Each recipient received 7.5×10^6 cells in PBS through a tail vein injection.

2.3. Behavior

Animals were trained on a food reinforcement schedule in operant chambers, following by surgery to implant the vein catheters, during which blood samples were obtained (see Fig. 1A).

2.3.1. Acquisition and maintenance of self-administration:

Animals were trained over 21 daily sessions to press a lever to receive cocaine (1 mg/kg/infusion in 100 µl, i.v.), on a FR1, 30 s time-out reinforcement schedule (Miguéns et al., 2015, 2011).

2.3.2. Extinction of cocaine self-administration:

The animals underwent extinction from day 22 until they reached the extinction criterion within 3 weeks: three consecutive days performing ≤ 20% of the average active lever (AL) presses recorded on days 19–21. During extinction, cocaine was replaced with the vehicle, injected i.v. after pressing the AL under the same schedule indicated previously.

2.3.3. Reinstatement of drug-seeking behavior:

Following extinction, the reinstatement of drug-seeking behavior was tested with a sub-threshold priming injection of cocaine (7.5 mg/kg, i.p.). During the reinstatement session, the extinction conditions were maintained and after the session, the animals were sacrificed to obtain brain, spleen and blood samples.

2.4. Flow cytometry

Four-color flow cytometry was performed to identify GFP^+ BM-derived cells (BMDCs), focusing on adaptive T- and B-cells and innate immune cells, such as NK-cells, monocytes and granulocytes (Table S1). We discriminated $CD4^+$ and $CD8^+$ T-cells and also focused on $CD4^+CD25^+$ T-cells since they can synthesize, store and release dopamine (Cosentino et al., 2007). Following erythrocyte lysis, 1×10^6 leukocytes were incubated for 30 min at 4 °C in the dark with 1 µg of anti-rat antibodies. After washing, the cells were fixed and analyzed.

2.5. Immunohistochemistry

Indirect dual or triple immunofluorescence was performed to detect BMDCs in the brain of F344/LEW-BM rats. Slices were incubated in PBS containing normal donkey serum (5%), Triton X-100 (0.2%) and the primary antibodies: polyclonal goat anti-GFP antiserum in combination with either a polyclonal rabbit anti-Iba1 antiserum or a monoclonal rabbit anti-CD3 antibody. After rinsing, antibody binding was detected with the corresponding fluorescent secondary antibodies diluted in PBS: Cy2-conjugated donkey anti-goat antibody and either Cy3- or Cy5-conjugated donkey antibodies. Before the antibodies were visualized, the slices were incubated with 4′-6-diamidino-2-phenylindole (DAPI) to counterstain the cell nuclei, rinsed, mounted and coverslipped with anti-fading medium.

2.6. qRT-PCR

Reverse transcription (RT) was carried out in a thermal cycler (Veriti, Applied Biosystems, Foster City, CA, USA) on 500 ng of the total RNA isolated as detailed in the Supplementary material. The cDNA obtained was used as the template for the qPCR and amplified with the primers of interest (Table S2) using the SYBR-Green method on a QuantStudio 7 Flex device (Applied Biosystems). The *GAPDH* gene was used for

normalization.

2.7. Data analysis

For comparison, two tailed Student's *t*-tests were applied unless otherwise specified. Leukocyte subpopulations were analyzed using a two-way repeated measures analysis of variance (ANOVA), with the BM transplant (F344 or LEW) as the between-subject factor and the treatment (basal or post-cocaine) as the within-subject factor. A Factorial Analysis (FA) was performed to detect possible relationships among the changes in gene expression and the experimental groups. A Principal Component Analysis was also used to extract components when multiple genes were analyzed. Statistical tests were performed using either GraphPad.7 or SPSS 22.0 for Windows.

3. Results

3.1. Transplantation and integration of BMDCs

In both groups, transplantation of BMDCs was associated with a similar survival rate 100 days after transplantation (90% for F344/F344-BM rats and 85% for F344/LEW-BM rats; Chi squared = .096; $p = .76$; Fig. S1), the time point from which the animals were considered to be included in the experiment having reached 175–225 g body weight. In these analyses, we studied F344/LEW-BM rats in which there was a continuous increase in the relative proportions (referred to hereafter as %) of GFP^+ donor-derived blood cells (Fig. 1B, C), as well as F344/F344-BM rats that maintained the usual leukocyte profile for the F344 strain (see Fig. S2). Thus, at the time of surgery the values for each of the leukocyte subsets were considered as the basal levels, and they were compared between the groups to confirm the success of the transplant. At baseline, the F344/LEW-BM rats displayed a similar immune profile to that described for LEW rats (Fig. S2; Griffin and Whitacre, 1991; Ucha-Tortuero, 2012), with a higher % of T-cells, a lower % of B-cells and a higher T/B-cell ratio (Figs. 1D-E, and S3) than the F344 controls. Furthermore, F344/LEW-BM rats had higher % of $CD4^+$ T-cells and granulocytes, yet a lower % of $CD4^+CD25^+$ T-cells than the controls (Figs. 1D, S4 and S6). There were no differences in both groups regarding the % of $CD8^+$ T-cells, the $CD4^+/CD8^+$ T-cell ratio, the % of NK cells or that of monocytes (Figs. 1D, E, S4–S6). These data confirm the efficacy of our procedure in reconstituting the ablated BM with the transplanted cells.

3.2. LEW-BM transfer induces the reinstatement of cocaine-seeking behavior in F344 rats

During the self-administration period, F344/F344-BM and F344/LEW-BM rats delivered more active lever press scores (ALPS) than inactive lever press scores (ILPS: Fig. 2A and C), with no differences in the number of injections per session (Fig. 2A) or the total amount of cocaine consumed (Table S3). No differences were observed in the days to fulfill the extinction criterion, with a similar number of ALPS and ILPS in the last extinction session in both the groups (Fig. 2B-C and Table S3). However, during the reinstatement session, F344/LEW-BM rats produced more ALPS than ILPS, while no differences were observed in F344/F344-BM rats (Fig. 2C). In the light of this difference, we further compared the ALPS performed during the reinstatement session with those on the last day of extinction for each group as described previously (Miguéns et al., 2013), which only differed significantly in F344/LEW-BM rats ($t_{(5)} = 4.043$, $p = .0099$; data not shown). These data demonstrated that only the F344 rats transplanted with LEW BM reinstated cocaine-seeking behavior. Finally, there were no signs of graft-vs-host disease (GVHD) in F344/LEW-BM rats throughout the experiment: no diarrhea, no hair loss and no weight loss compared to the F344/F344-BM rats (Figure S7).

3.3. Integration of BMDCs into brain

The integration of BMDCs into the brain of recipients depends on the experimental conditions, and it may involve glia, neurons and round leucocyte-like cells (Díaz et al., 2015; Recio et al., 2011). We sacrificed F344/LEW-BM rats at P155-170, sufficiently long after transplantation to be able to detect a considerable number of BM-derived neurons in their brains (Díaz et al., 2015). Yet in contrast to previous findings (Díaz et al., 2015; Recio et al., 2011), only microglia ($Iba1^+$) and round GFP^+ cells were detected, cells that appeared to mainly integrate into the meninges and choroid plexus, with only a minor presence in the parenchyma (Figs. 3 and S8). Moreover, no preference towards integration into addiction-related areas was observed (i.e. prefrontal cortex, striatum, hippocampus, basal ganglia, amygdala, or ventral tegmental area: Hyman et al., 2006). Thus, taking into account the small number of GFP^+ elements and their distribution, no further cell quantification was performed.

In terms of microglial cells, no apparent differences in their distribution or morphology were detected between the two experimental groups. In addition, no specific changes in these cells were observed relative to standard situations (Fig. S8A). That is, the shape, density and distribution of microglia were consistent with features of healthy brains, differing from the amoeboid profile or the regional confluence of microglia in pathological situations (Baltanás et al., 2013; Díaz et al., 2011).

The round GFP^+ cells were characterized by CD3 immunostaining revealing a small number of T-cells (Figs. 3D and S8B), poorly integrated into the choroid plexus (Fig. S8C). Finally, a few round GFP^+ $CD3^-$ cells were also detected (Figs. 3D, S8B, and C).

3.4. Cocaine induces different effects on F344- and LEW-derived leukocytes

The history of exposure to cocaine (including cocaine self-administration and the challenge dose received following extinction) may differentially affect F344 or LEW-BMDCs. Thus, cocaine treatment (factor treatment: basal or post-cocaine), as well as the effect of transplanted BM (F344 or LEW), were analyzed by repeated measures ANOVA. Additionally, the post-cocaine data were expressed relative to the baseline (Fig. S9). The data obtained regarding cocaine treatment could be grouped into five categories: I) a similar effect in both groups; II) an effect on the F344/F344-BM rats; III) an effect on the F344/LEW-BM rats; IV) opposite effects between the two groups; and V) no effects.

In terms of category I, lower % of B-cells was found in both groups, such that the basal differences were maintained (Figs. 4, S3 and S9). In category II, cocaine decreased the % of $CD4^+$ T-cells but increased that of $CD8^+$ T-cells in F344/F344-BM rats, reducing the $CD4^+/CD8^+$ T-cell ratio. The differences in these parameters observed at baseline persisted in the two groups after treatment (Figs. 4, S4 and S9), although interactions of these factors were detected in all cases and no further comparisons were possible. The % of NK cells increased after cocaine treatment in F344/F344-BM rats, although this value was no different to that observed in the F344/LEW-BM rats (Figs. 4, S5 and S9). In category III, a higher % of T-cells was observed in F344/LEW-BM rats, causing a marked rise in the T/B-cell ratio (Figs. 4, S3 and S9), although an interaction was detected in the latter. On other hand, the % of $CD4^+CD25^+$ T-cells in F344/LEW-BM animals interestingly fell after treatment. These three parameters differed between the group's post-cocaine consumption (Figs. 4, S4 and S9). In category IV, an opposite effect of cocaine was observed on granulocytes, with a higher % in F344/F344-BM rats and a lower % in F344/LEW-BM rats, in contrast to the baseline values (Figs. 4, S6 and S9). In category V, cocaine did not affect the % of monocytes in either group (Figs. 4, S6 and S9).

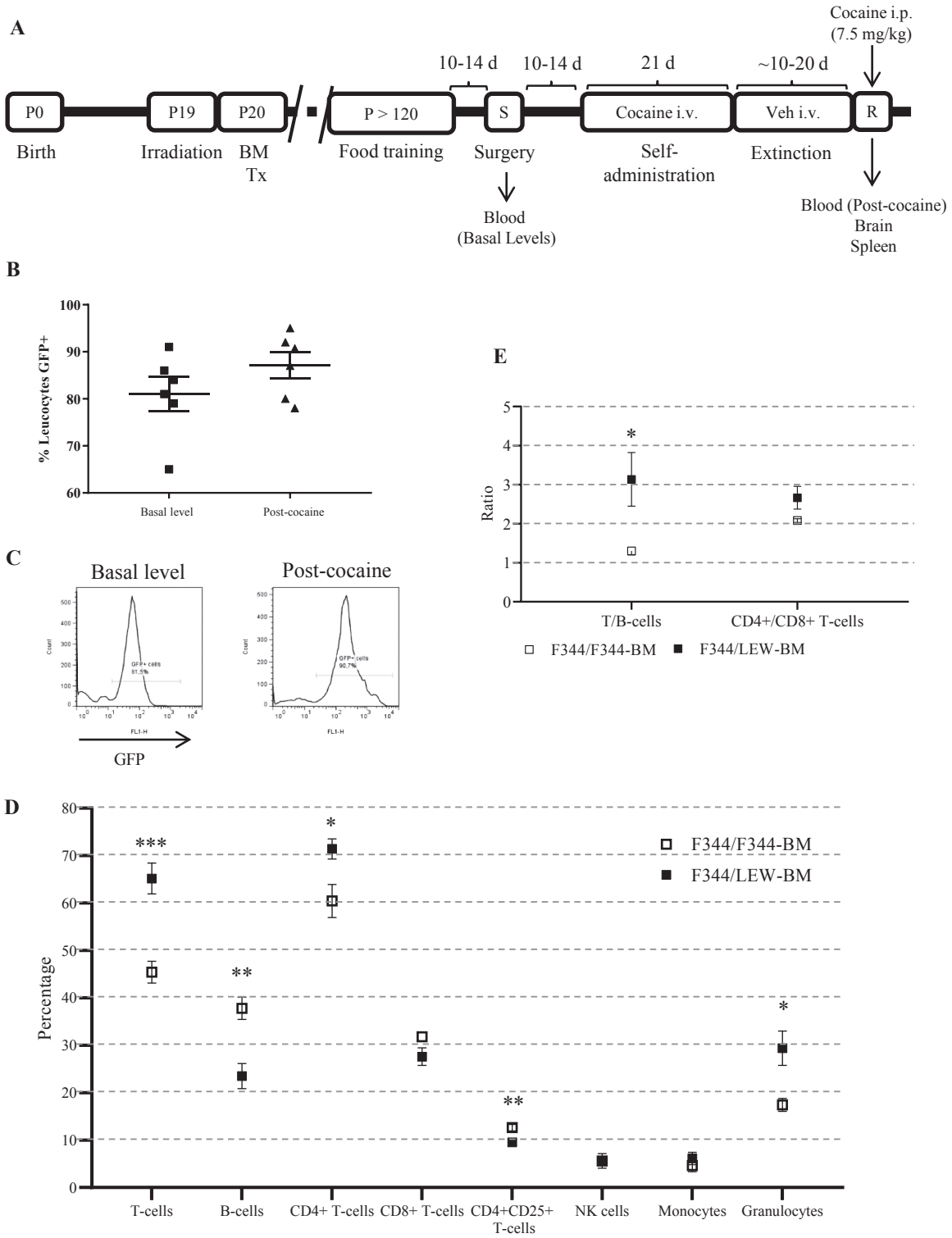

Fig. 1. Timeline of the experimental procedures (A) and flow cytometry analysis of bone marrow-derived cells. There was an increase in GFP^+ leukocytes in the peripheral blood of Fischer 344 ($GFP^{-/-}$) rats transplanted with bone marrow from Lewis rats ($GFP^{+/+}$) over time (81% ± 3.62 basal levels, and 87% ± 2.80 post-cocaine, B), as seen in representative histograms showing GFP^+ (FL1) leukocytes in peripheral blood (C). The values were obtained on the day of surgery, 10–14 days before commencing the self-administration protocol (basal level). The samples were also analyzed to study the peripheral blood subpopulations (D): $CD3^+CD45RA^-$ lymphocytes (T-cells) relative to the total lymphocytes ($t_{(11)} = 5.061$, $p = .0004$); $CD3^-CD45RA^+$ lymphocytes (B-cells) relative to the total lymphocytes ($t_{(9)} = 3.946$, $p = .0034$); $CD3^+CD4^+$ lymphocytes ($CD4^+$ T-cells) relative to the T-cells ($t_{(10)} = 2.421$, $p = .036$); $CD3^+CD8^+$ lymphocytes ($CD8^+$ T-cells) relative to the T-cells; $CD3^+CD4^+CD25^+$ lymphocytes ($CD4^+CD25^+$ T-cells) relative to the T-cells ($t_{(8)} = 3.356$, $p = .01$); $CD3^-CD161a^+$ lymphocytes (NK cells) relative to the total lymphocytes; $CD11b/c^+$ monocytes (Monocytes) relative to the leukocytes; and $CD11b/c^+$ granulocytes (Granulocytes) relative to the leukocytes ($t_{(8)} = 3.085$, $p = .015$). The ratios between the peripheral T-cells and B-cells ($t_{(9)} = 2.398$, $p = .04$), and between $CD4^+$ T-cells and $CD8^+$ T-cells, were also assessed (E). P, post-natal day; BM Tx, Bone marrow transplantation; S, Surgery; Veh, Vehicle; R, Reinstatement of drug-seeking behaviour; d, day/s; F344/F344-BM, Fischer 344 rats transplanted with bone marrow from Fischer 344 rats; F344/LEW-BM, Fischer 344 rats transplanted with bone marrow from Lewis rats. The data are expressed as the mean ± SEM: $*p < .05$, $**p < .01$, $***p < .001$ unpaired Student's *t*-test compared to the F344/F344-BM control group.

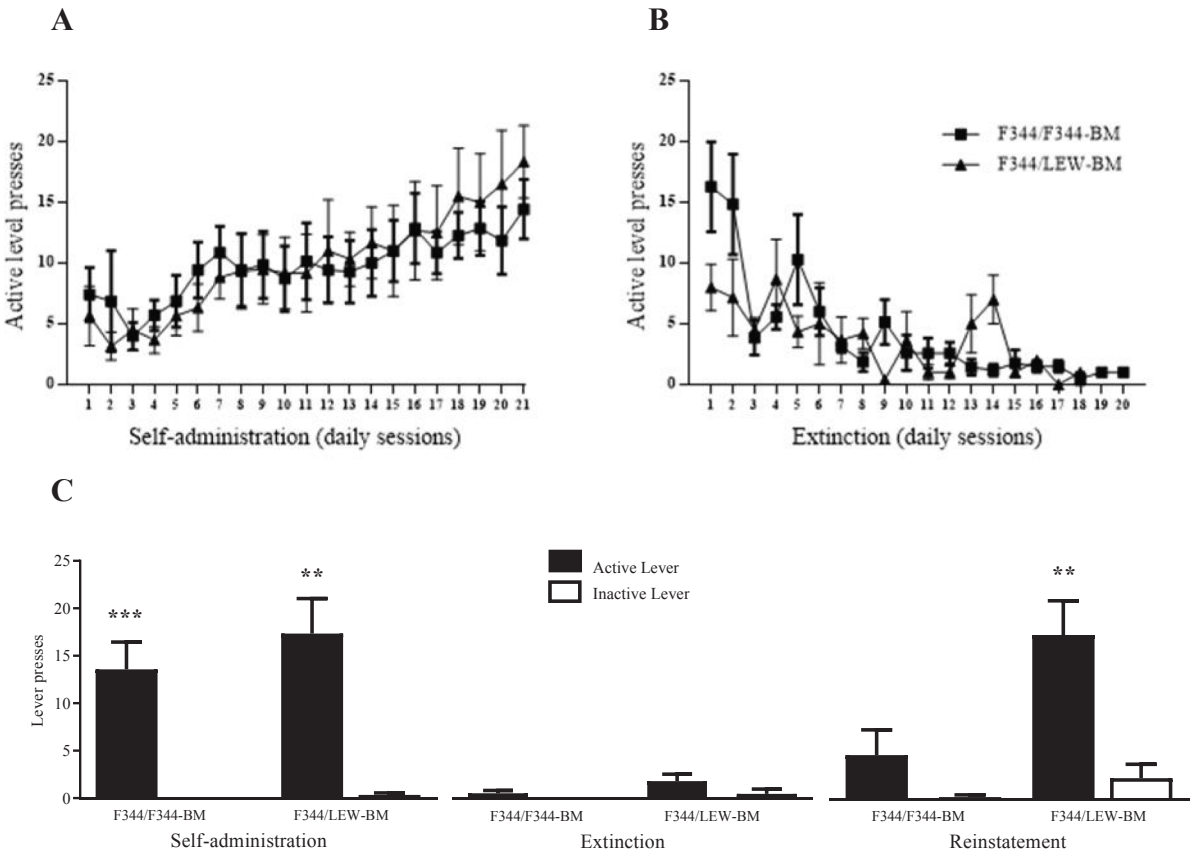

Fig. 2. Cocaine self-administration, extinction and reinstatement. A, Irradiated Fischer 344 rats transplanted with Fischer 344 bone marrow (F344/F344-BM, squares, n = 7) or with Lewis bone marrow (F344/LEW-BM, circles, n = 6) were trained to self-administer cocaine (1 mg/kg/infusion) during 21 days under a fixed ratio 1 (FR1) schedule. The daily sessions lasted 2 h. No significant differences were observed in cocaine self-administration (A) or extinction behavior (B) between F344/F344-BM and F344/LEW-BM rats. C, In the last self-administration session, both groups showed significant differences in the number of active and the inactive lever presses (C, left panel $t_{(12)} = 4.703$, $p = .0005$, F344/F344-BM; $t_{(10)} = 4.584$, $p = .001$, F344/LEW-BM: $^{**}p < .01$, $^{***}p < .005$ unpaired Student's *t*-test), differences that had disappeared by the last extinction session (C, middle panel). However, the cocaine priming injection (7.5 kg/mg, i.p.) in F344/LEW-BM rats was associated with more responses using the lever previously associated with cocaine (C, right panel: $t_{(10)} = 3.883$, $p = .003$, $^{**}p < .01$, unpaired Student's *t*-test). Reinstatement of cocaine-seeking behavior was not observed in F344/F344-BM rats, as reflected by the lack of statistically significant differences in this group between the number of active and inactive lever responses ($t_{(12)} = 1.657$, $p = .1233$, unpaired Student's *t*-test).

3.5. LEW-BM transfer induces a less immunosuppressive splenic environment in F344 rats after cocaine, associated with Th17 polarization and weaker innate signaling

Given that the behavior of F344 rats depended on the BMDCs transferred, it is notable that their immunological parameters are biologically related to the reinstatement data, albeit in a complex manner. This complexity is one reason why we performed a multi-dimensional analysis that allowed us examine the relationship between all of these complex factors. Furthermore, as the expression of multiple related genes was studied simultaneously, we used a FA as a multi-dimensional statistical test. This FA analysis did not reveal any relationship between the immune cell parameters and the reinstatement data, and therefore, we analyzed the immune-related genes. Bearing in mind the complexity of this multivariate analysis and to avoid confusion, only the significant results obtained are described below.

In this sense, considering the decrease in the $CD4^+CD25^+$ T-cells levels in LEW-BM transplanted rats, we evaluated the splenic expression of five genes related to these cells (Table S2). Initially, using the FA, we verified that the first two components explained 88.35% of the variance (Table 1.AI), which enabled the dimensionality of the data to be reduced from 5 (number of genes or initial dimensions) to 2 (components). Component 1 was related to the expression of *D_5*, *Foxp3* and *Ctla-4*, whereas the second component was mainly explained by *Il-10* and *Tgf-β1* expression (Fig. 5A and Table 1.AII). Thus, component 1 confirmed a close relationship amongst non-secreted proteins, while component 2 was related to secreted cytokines. Once these genes had been sorted into groups of related variables, a Mann-Whitney *U* test confirmed there were no differences among the genes of component 1, unlike the secreted cytokines due to the increase in *Tgf-β1* expression in F344/F344-BM relative to F344/LEW-BM rats (Fig. 5C).

The lower numbers of $CD4^+CD25^+$ T-cells and the weaker TGF-β1 expression suggested weaker immunosuppressive modulation, which might boost the expansion of effector T-cells in F344/LEW-BM rats. Thus, we evaluated the splenic expression of genes related to T helper 17 (Th17) cells (Table S2). Following a similar FA, two first components explained 93.68% of the variance in the data (Table 1.BI), which again enabled the dimensionality of the data to be reduced from 5 to 2. Component 1 was related to *Il-1β*, *Il-23* and *Tgf-β1* expression, while the second component was explained by *Il-17a* and *Il-6* expression (Fig. 5B and Table 1.BII). A Mann-Whitney *U* test revealed higher *Tgf-β1* and *IL-1β* expression in F344/F344-BM rats (Fig. 5C), probably related to an association with $CD4^+CD25^+$ T cells (see above) and innate immunity (see below), respectively. Regarding the second component, there was stronger expression of *Il-17a* in F344/LEW-BM rats, suggesting a predominant Th17 profile in these animals (Fig. 5C). An additional analysis plotted the gene expression data corresponding to the experimental rats in the first factorial plane, which supported our hypothesis of Th17

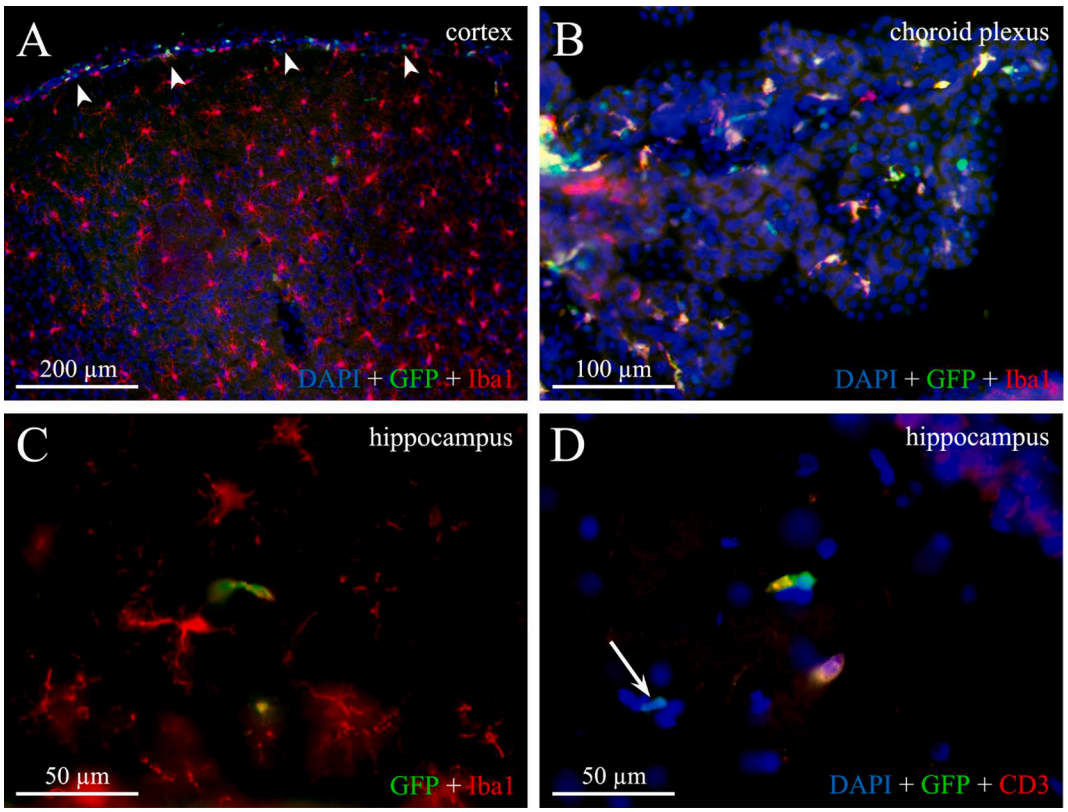

Fig. 3. Analysis of the transplant-derived cells in the brain of F344/LEW-BM rats. A, The vast majority of transplant-derived cells (GFP^+, green) appeared in the meninges (arrowheads), some of which were microglia (Iba1, red). B, The choroid plexus has a high density of transplant-derived microglial cells: GFP^+ (green) and $Iba1^+$ (red). C, D, A few bone marrow-derived cells (GFP^+) were evident in the hippocampus, some of which were microglial cells ($Iba1^+$, red: C) and others were round elements, either T-cells ($CD3^+$, red: D) or other immune-like cells (arrow). In A, B and D, all the cell nuclei were counterstained with DAPI (blue). Scale bar 200 µm for A, 100 µm for B, and 50 µm for C and D. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

polarization (Fig. S10).

Finally, innate immunity was evaluated by considering *Tlr-2*, *Tlr-4* and *Il-1β* expression using the classic non-parametric Mann-Whitney *U* test. These three genes were expressed more strongly in F344/F344-BM animals (Fig. 5C), highlighting a strong reduction in the innate signaling after LEW BM transfer. Furthermore, the relative splenic weight was higher in F344/LEW-BM rats (7.25 ± 0.77 mg/g) than in their control counterparts (4.12 ± 0.39 mg/g; Fig. S11).

4. Discussion

We demonstrate here that LEW BM transplantation into F344 rats produces a shift in the rat's immune cell profile and in their reinstatement of cocaine-seeking behavior, both resembling that of LEW rats (Miguéns et al., 2013; Ucha-Tortuero, 2012). LEW-BM transfer augmented the number of T-cells and the T/B-cell ratio, which was further enhanced following cocaine exposure, as reported previously (Gan et al., 1998; Lax et al., 2018; Levandowski et al., 2016). Our findings suggest that T-cells, and particularly the $CD4^+$ T-cell response triggered by cocaine re-exposure, may underlie this behavioral change. A $CD4^+$ T-cell response polarized to a Th17 profile could be activated by recognizing cocaine, favored by a dampened influence of immunosuppressive elements like $CD4^+CD25^+$ T-cells, TGF-β1 and IL-10. In addition, stronger D_5 dopaminergic signaling in lymphocytes might also participate in this phenomenon.

The effects of cocaine in the CNS have been widely studied (Ritz et al., 1990) and it is known to influence immunity through peripheral pathways (Bhowmick et al., 2009; Marasco et al., 2014; Nistico et al., 1994). Moreover, as catecholamine receptors and membrane transporters are widespread in immune cells (Amenta et al., 2001, 1999; Cosentino et al., 2002; Matt and Gaskill, 2020; Mignini et al., 2009; Ricci and Amenta, 1994), immunocytes may be directly stimulated by sustained catecholamine levels on exposure to cocaine. Cocaine may also be recognized as a foreign substance by leukocytes, activating adaptive and innate immunity. Indeed, we previously found that cocaine self-administration produces a striking increase in spleen size, as described elsewhere (Kubera et al., 2008), and in the splenic leukocyte count in LEW rats, augmenting the T/B-cell ratio as seen here (Assis et al., 2020). These events resemble the splenic proliferative T-cell response triggered by blood-borne substances (e.g. cocaine), which is captured by splenic antigen-presenting cells (APCs) to be presented to and recognized by $CD4^+$ T-cells. This immune recognition of cocaine by T-cells could have long-lasting consequences, especially in terms of relapse.

The lower levels of $CD4^+CD25^+$ T-cells detected in F344/LEW-BM rats were consistent with their weaker *Tgf-β1* expression, accompanied by a reduction in *Il-10* and a tendency towards stronger splenic D_5 expression. These data is consistent with D1-type receptor activation limiting the synthesis of these immunosuppressive cytokines (Cosentino et al., 2007; Ferreira et al., 2014; Kipnis et al., 2004; Levite, 2016). Since cocaine may heighten peripheral dopaminergic tone, enhanced D_5 signaling in LEW-derived cells could further inhibit the synthesis of these cytokines by $CD4^+CD25^+$ T-cells ("inhibition of inhibition": Cosentino et al., 2007; Kipnis et al., 2004). In line with cocaine inducing a less immunosuppressive state, there is less IL-10 after cocaine reinstatement, in conjunction with an increase in $CD4^+$ and $CD8^+$ T-cells (Fox et al., 2012; Gan et al., 1998; Kubera et al., 2008; Moreira et al., 2016). Indeed, the $CD4^+/CD8^+$ T-cell ratio in Wistar rats increased following cocaine consumption (Jankowski et al., 2010), a similar

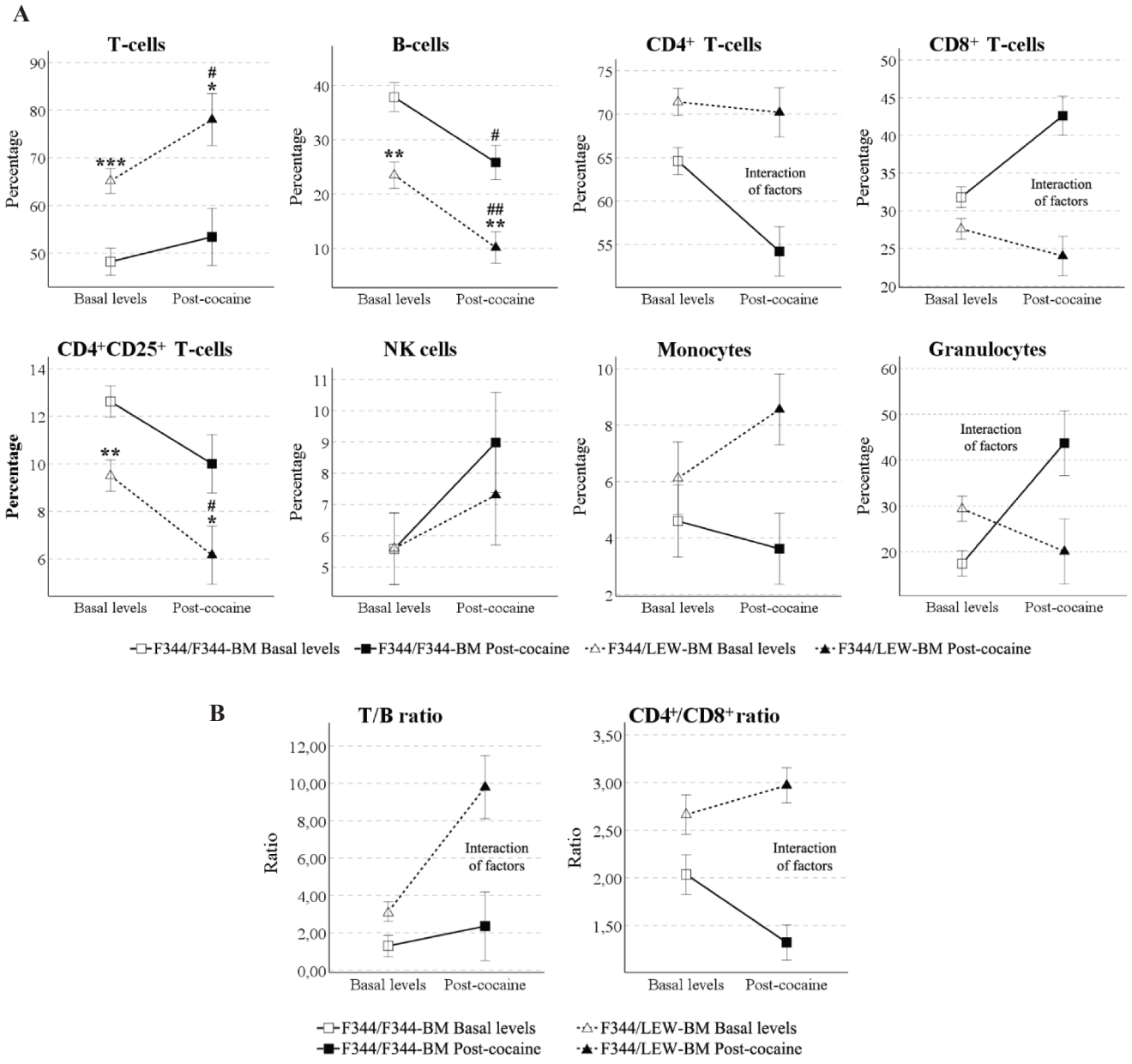

Fig. 4. Flow cytometry analysis of BMDCs from F344 rats transplanted with F344 (F344/F344-BM) or Lewis (F344/LEW-BM) BM after the reinstatement session. The values were compared with those obtained on the day of surgery, before starting the self-administration protocol (Basal Levels: BL), to evaluate the response to a history of cocaine treatment (self-administration and cocaine challenge dose: Post-cocaine, PC). The samples were analyzed to study the peripheral blood subpopulations (A): $CD3^+CD45RA^-$ lymphocytes (T-cells) relative to the total lymphocytes (repeated measures ANOVA BL/PC $F = 5.631$, $p = .042$; $t_{(11)} = 5.061$, $p = .0004$, BL; and $t_{(9)} = 3.036$, $p = .0141$, PC; repeated measures ANOVA for rat groups $F = 16.726$, $p = .003$; $t_{(5)} = 3.987$, $p = .0105$, F344/LEW-BM rats); $CD3^-CD45RA^+$ lymphocytes (B-cells) relative to the total lymphocytes (repeated measures ANOVA BL/PC $F = 36.020$, $p = .000$; $t_{(9)} = 3.946$, $p = .0034$, BL; and $t_{(9)} = 3.637$, $p = .0054$, PC; repeated measures ANOVA for rat groups $F = 19.733$, $p = .002$; $t_{(4)} = 3.586$, $p = .0231$, F344/F344-BM rats; and $t_{(5)} = 5$, $p = .0041$, F344/LEW-BM rats); $CD3^+CD4^+$ lymphocytes ($CD4^+$ T-cells) relative to the T-cells (repeated measures ANOVA showed interaction of factors $F = 4.600$, $p = .064$); $CD3^+CD8^+$ lymphocytes ($CD8^+$ T-cells) relative to the T-cells (repeated measures ANOVA showed interaction of factors $F = 9.690$, $p = .014$); $CD3^+CD4^+CD25^+$ lymphocytes ($CD4^+CD25^+$ T-cells) relative to the T-cells (repeated measures ANOVA BL/PC $F = 9.600$, $p = .015$; $t_{(8)} = 3.356$, $p = .01$, BL; and $t_{(9)} = 2.612$, $p = .0282$, PC; repeated measures ANOVA for rat groups $F = 12.110$, $p = .008$; $t_{(4)} = 4.572$, $p = .0102$, F344/LEW-BM rats); $CD3^-CD161a^+$ lymphocytes (NK cells) relative to the total lymphocytes; $CD11b/c^+$ monocytes (Monocytes) relative to the leukocytes; and $CD11b/c^+$ granulocytes (Granulocytes) relative to the leukocytes (repeated measures ANOVA showed interaction of factors $F = 14.127$, $p = .006$). Moreover, the ratios between the peripheral T-cells and B-cells (repeated measures ANOVA showed interaction of factors $F = 5.420$, $p = .045$), and the $CD4^+$ T-cells and $CD8^+$ T-cells (repeated measures ANOVA showed interaction of factors $F = 4.738$, $p = .061$) were obtained (B). The data are expressed as the mean ± SEM: $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ unpaired Student's *t*-test compared to the F344/F344-BM control group; $^{\#}p < .05$, $^{\#\#}p < .01$ paired Student's *t*-test compared to the BL.

response as that of F344/LEW-BM rats. Thus, clinical and experimental data support our hypothesis that cocaine triggers an effector $CD4^+$ T-cell response, boosted by a less immunosuppressive environment in LEW-derived lymphocytes.

We had expected to detect differences in *Foxp3* and *Ctla-4* mRNA expression between the experimental groups, although a recent paradigm shift regarding T-cell lineage development highlights the plasticity of Treg cells and their potential to paradoxically convert into highly pro-

Table 1
Reduction of dimensionality using the PCA as extraction method (I, left) and the associated rotated component matrices showing the results of the FA (II, right), for the $CD4^+CD25^+$ T-cell related genes (A) and Th17-related genes (B). Note that the two first components (shadowed lines, I, left) explain the 88.345 % and the 93.674 % of the variance (that is to say, much more than the 75 %) for A and B, respectively, thus allowing the reduction of our data to a bidimensional space (plane) in both analyses. Each rotated component matrix explains the contribution of each variable to the variance of these two first components of the bidimensional space (shadowed lines, II, right). Note that the five genes in A and B (variables; II, right side) are clearly sorted in both components (i.e. contribution values close to 1 for one component and close to 0 for the another). Contributions lower than 0.3 have been softened on the rotated component matrices. Comp., component; cumul., cumulative.

A

	I. Total variance explained									II. Rotated component matrices [a]				
	Initial autovalues			Extraction sums of squared loadings			Rotation sums of squared loadings			Variables ($CD4^+CD25^+$ T-cell related genes)				
Comp.	Total	% of variance	Cumul. %	Total	% of variance	Cumul. %	Total	% of variance	Cumul. %	*Ctla-4*	*D5*	*Foxp3*	*Il-10*	*Tgf-β1*
1	2.799	55.977	55.977	2.799	55.977	55.977	2.523	50.458	50.458	0.922	0.934	0.833	-0.117	-0.096
2	1.618	32.367	**88.345**	1.618	32.367	**88.345**	1.894	37.887	**88.345**	0.017	-0.207	-0.135	0.955	0.959
3	0.320	6.402	94.747											
4	0.161	3.212	97.959											
5	0.102	2.041	100.000											

B

	Initial autovalues			Extraction sums of squared loadings			Rotation sums of squared loadings			Variables (Th17-related genes)				
Comp.	Total	% of variance	Cumul. %	Total	% of variance	Cumul. %	Total	% of variance	Cumul. %	*Il-1β*	*Il-6*	*Il-17a*	*Il-23*	*Tgf-β1*
1	2.765	55.309	55.309	2.765	55.309	55.309	2.733	54.658	54.658	0.973	0.198	-0.120	0.958	0.903
2	1.918	38.365	**93.674**	1.918	38.365	**93.674**	1.951	39.016	**93.674**	-0.022	0.979	0.992	0.041	0.077
3	0.259	5.185	98.858											
4	0.055	1.105	99.964											
5	0.002	0.036	100.000											

inflammatory Th17 cells (Bovenschen et al., 2011; Remedios et al., 2018). $FoxP3^+$ cells from individuals with autoimmune diseases can easily differentiate into IL-17A producing cells as FoxP3 expression is progressively lost ("Treg instability": Du et al., 2014). These $IL\text{-}17A^+FoxP3^+$ T-cells may also retain the expression of other Treg-related proteins like CTLA-4 (Du et al., 2014). We found higher *Il-17a* splenic expression in F344/LEW-BM rats coincident with high levels of *Il-6* but less *Il-23* and *Il-1b* mRNA (the expression of which varies together with *Tgf-β1* and is lower in F344/LEW-BM rats). These data suggest that a Th17 subset, probably induced by an IL-6-dependent mechanism, may be the effector T-cells responsible for recognizing cocaine after LEW-BM transfer. Some Th17 cells may derive from $FoxP3^+$ cells that also express CTLA-4, and that progressively transform into IL-17A-producing cells in F344/LEW-BM rats. Our data strongly agree with evidence that dopamine stimulates the expansion of IL-17-producing T-cells in autoimmune diseases through its ability to induce IL-6 production by monocytes and $CD4^+$ T-cells (Ferreira et al., 2014). Thus, this Th17 polarization in LEW-BMDCs might be due to Treg instability and/or stronger D_5 signalling, possibly also explaining the high incidence of autoimmunity in LEW rats (Wilder et al., 2000). A Th-17 profile could also be induced in F344/LEW-BM rats by minor histocompatibility antigens, which differed in both strains, although the low mortality rate (similar in both groups) and the absence of signs of GVHD incline us to rule out this possibility, even more so if we consider the vulnerability of LEW rats to autoimmunity described previously. Indeed, we suggest that the Treg/Th17 balance (immunosuppression/pro-inflammation) is skewed towards an activated state in LEW-derived lymphocytes, contrasting with the status of F344-derived cells. This is consistent with LEW rats having fewer inhibitory cytokines than F344 rats, with their immunocytes maintained more efficiently in a proliferative state (Mattapallil et al., 2008). Finally, as cocaine further reduced the $CD4^+CD25^+$ T-cells in F344/LEW-BM rats, these data could also explain the mild autoimmune response reported after cocaine exposure (Trimarchi et al., 2013).

Regarding innate immunity, several studies have focused on cocaine and microglia (Clark et al., 2013; Hutchinson and Watkins, 2014). Although no association was found between this drug and microglial stimulation (Narendran et al., 2014), addiction was recently proposed to be a consequence of innate immune activation in the brain (Crews et al., 2011). This theory and our T-cell hypothesis are not mutually exclusive, since both innate and adaptive immunity might act in parallel, influencing each other to ultimately modulate behavior. This idea is supported by recent evidence regarding IL-17A as an activator of microglia in Parkinson's disease (Liu et al., 2019b). Cocaine can activate TLR-4 and TLR-2 on microglia (Liao et al., 2016; Northcutt et al., 2015), and perhaps also on peripheral innate immunocytes (including APCs). Our data demonstrates stronger *Tlr-4*, *Tlr-2* and *Il-1β* mRNA expression in F344-BMDCs, which together with the increased granulocyte levels suggests that the F344 strain may mount a stronger innate response to cocaine, while its adaptive immunity might be limited by enhanced immunosuppression. Concordantly, increased TLR-4 expression by monocytes promotes $CD4^+CD25^+$ T-cell differentiation (Hao et al., 2017). In parallel, the activation of TLRs on APCs improves the effectiveness of antigen presentation (Abbas et al., 2017). Thus, if cocaine is haptenized and presented by APCs to T-cells in the spleen, this process could be exaggerated by cocaine activating TLR-4 and TLR-2 in parallel, even in LEW-derived cells expressing lower levels of TLRs. Such a mechanism might underlie the enhanced antigen presentation provoked by cocaine and reported some 20 years ago (Shen et al., 1999). In conjunction, splenic activation induced by cocaine (involving TLRs, APCs and $CD4^+$ T-cells) might explain the splenomegaly we and others have observed (Khan et al., 2017; Kubera et al., 2008). Indeed, F344/LEW-BM rats develop a relatively heavier spleen than their control counterparts. Together these data reinforce the idea that the balance towards an innate immune response would predominate over the adaptive response in F344/F344-BM rats. By contrast, the latter would predominate in F344/LEW-BM rats, underlying immune-mediator release that induces behavioral reinstatement.

The influence that $CD4^+$ T-cells can exert on behavior arises from cytokines that cross the BBB and/or by their transmigration into the CNS (Filiano et al., 2017; Prinz and Priller, 2017), which can affect several

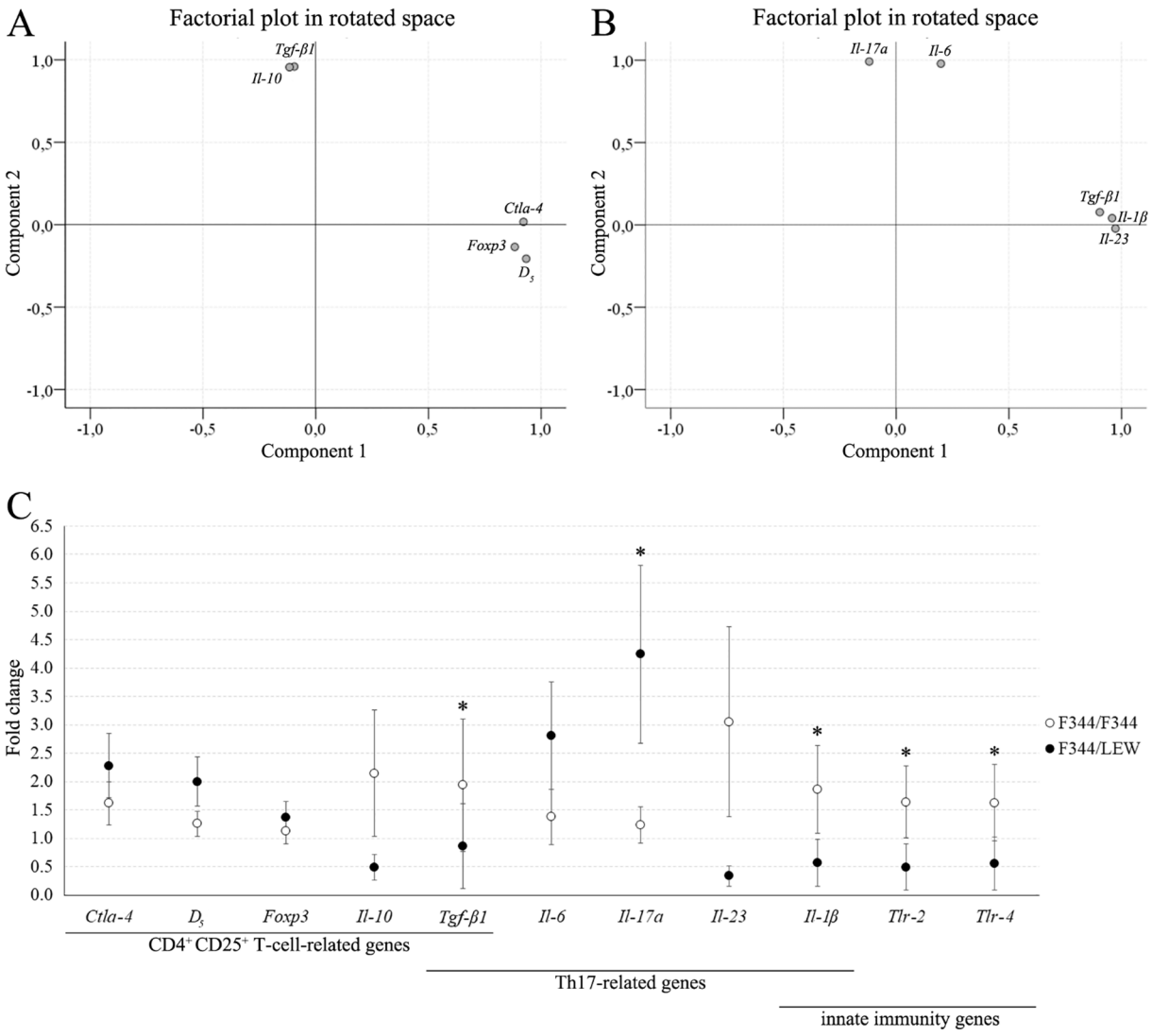

Fig. 5. Analysis of gene expression. A, First factorial plane in rotated space displays the relationship amongst the characteristic genes of $CD4^{+}CD25^{+}$ T-cells. Note that *Ctla-4*, D_5 and *Foxp3* (genes encoding two membrane receptors and a transcription factor, i.e. non-secreted proteins) have a clear distribution in the first component of the FA, whereas *Tgf-β1* and *Il-10* (secreted cytokines) are distributed in the second component. B, First factorial plane in the rotated space showing the relationship amongst representative genes of Th17 cells. Here, *Il-1β*, *Il-23* and *Tgf-β1* (genes associated with Th17 polarization that share activities with other immunological processes) have a clear distribution in the first component of the FA, whereas *Il-6* and *Il-17a* (genes involved in Th17 polarization and secretion, respectively) are distributed in the second component. C, The graphs showing the change in expression of the eleven genes analysed, sorted by immunological cell type or process. Note that *Tlr-2* ($p = .022$), *Tlr-4* ($p = .035$), *Tgf-β1,* ($p = .035$) and *Il-1β* ($p = .035$) are all expressed more strongly in F344/F344-BM rats, whereas *Il-17a* ($p = .014$) is expressed more strongly by F344/LEW-BM rats (*$p < .05$, Mann-Whitney *U* test).

brain processes (e.g. social behaviour, spatial learning and memory) and may involve plasticity in T-cell responses (Filiano et al., 2016; Kipnis and Filiano, 2018; Korn and Kallies, 2017; Subramanian et al., 2001; Wilson et al., 2010; Yirmiya and Goshen, 2011). The recruitment of T-cells to the CNS is partially understood as they can migrate across the choroid epithelium and cross the BBB when it is altered, while vascular channels connecting the skull BM and brain have also recently been described (Herisson et al., 2018; Korn and Kallies, 2017; Strazielle et al., 2016; Wilson et al., 2010). Furthermore, psychostimulants can alter BBB permeability (Davidson et al., 2018; Kousik et al., 2012). Nevertheless, we found GFP^{+} cells to be scarce in the brain of F344/LEW-BM rats and hence, our findings suggest a more prominent peripheral immune influence on reinstatement (e.g. IL-17A released peripherally) rather than an effect of T-cells that have transmigrated into the brain. Interestingly, IL-17A was recently related to dopaminergic dysfunctions in Parkinson's disease (Liu et al., 2019b). A peripheral influence associated with cocaine relapse was proposed when cocaine methiodide, which cannot cross the BBB, induced reinstatement in animals that had previously self-administered cocaine (Wang et al., 2013a; Wise et al., 2008). This component was attributed to interoceptive keys that "*precede and predict the rewarding action of cocaine in experienced users*" and that can alter the activity of ventral tegmental area (Mejías-Aponte and Kiyatkin, 2012; Wise et al., 2008). In the light of the current findings, these effects might be explained by $CD4^{+}$ T-cells releasing mediators after recognizing cocaine methiodide peripherally. Further studies will be necessary to confirm the neuro-immune connexions underlying these phenomena.

Repeated cocaine exposure produces lasting changes in neural circuits that underlie addiction (Everitt and Robbins, 2016; Guillem and Ahmed, 2018; Higuera-Matas et al., 2011). One consequence of this plasticity is the behavioral and incentive-motivational sensitization that follows re-exposure to cocaine (Bickel et al., 2018; Robinson and Berridge, 2008, 1993; Robinson and Kolb, 2004; Vezina, 2004; Wang et al., 2013b), which is distinct in LEW and F344 rats (Kosten et al., 1994). Interestingly, the phenomenon of sensitization is not exclusive to the

CNS but it has also been widely described at the immune level, with similarities regarding the intermittent temporal patterns of exposure to a stimulus (Bauer et al., 2018; Calipari et al., 2015, 2013; Ort et al., 2019). Furthermore, antigen memory is a cardinal feature of adaptive immunity, closely related to sensitization. Upon encountering an antigen, T-cells differentiate into heterogeneous populations, among which a given subset mature into memory T-cells to provide long-term immunity (Chang et al., 2014; Rosenblum et al., 2016). Thus, while cocaine addiction is indisputably a progressive process provoked by CNS mechanisms, it is possible that sensitization and memory phenomena towards cocaine also exist in $CD4^+$ T-cells. Such immune mechanisms could contribute to the behavioral and incentive-motivational sensitization, and especially, to the highly efficient and unusually stable memory of this drug.

5. Conclusions

We demonstrate here that the reinstatement of drug-seeking behavior in a resistant rat strain can be changed by transplanting BM cells from a vulnerable strain. We propose a peripheral immune mechanism that might act in parallel to the central effects of cocaine exposure, mainly mediated by the generation of a subset of $CD4^+$ T-cells (Th17) capable of recognizing cocaine and generating memory to it. The long-term persistence of memory T-cells and their activation following subsequent cocaine exposure would work with lifetime memory, similar to how a vaccine works. Cocaine priming would trigger the release of neuro-immune mediators (e.g. IL-17A), interoceptive peripheral signals that drive relapse in the CNS. This long-term immune memory (potentially lifelong) could explain the long-term vulnerability to relapse in addicted individuals, despite long periods of detoxification. Furthermore, it is possible that in resistant individuals (e.g. F344 rats), the boosting of this adaptive immune signaling by cocaine would be silenced by a strong immunosuppression and a strong innate immune response. The dampened D_5 receptor expression by the F344-BMDCs might contribute to this resistance. In parallel, individuals genetically predisposed to Th17 polarization, probably due to higher Treg inhibition and/or instability (e.g. LEW rats), could generate strong adaptive immune memory to the drug, perhaps underlying relapse. Thus, the individual's predisposition to mount a given immune response after re-exposure to cocaine might explain their vulnerability to relapse. The present work opens up new perspectives to clinically manipulate the immune system (i.e.: targeting the Treg/Th17 balance), as proposed for other CNS disorders (Laumet et al., 2018), and to intervene with new strategies to treat addictions. Nevertheless, further experiments will still be necessary to unravel the complex processes that underlie the immune response and its cross-talk with central mechanisms in cocaine addiction.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.11.039.

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