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Characterization of CXCR5$^+$ CD8$^+$ T-cells in humanized NSG mice

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ABSTRACT

Humanized NOD/SCID/IL-2 receptor γ-chain^{null} (huNSG) mice recapitulate some features of human T-cell populations that can be exploited in basic and pre-clinical research. CXCR5$^+$ T CD8$^+$ T-cells play an important role in the control of viral infections and tumors. Indeed, they have been associated with low-level HIV replication, making them a possible novel correlate of protection, and potentially useful in the eradication of HIV reservoirs. Here, by flow cytometry, we evaluated the reconstitution of CXCR5$^+$ CD8$^+$ T-cells in huNSG mice engrafted with CD34$^+$ hematopoietic stem cells. This population was readily generated in huNSG mice, and where particularly confined to spleen and lymph nodes. These cells exhibited a follicular-like phenotype, with expression of Programmed Death (PD)-1, Inducible T-cell costimulatory (ICOS), and absence of CCR7. Moreover, CXCR5$^+$ CD8$^+$ T-cells had a higher expression of interleukin (IL)-21 and a higher cytotoxic potential compared with CXCR5$^-$ cells. HIV infection did not affect the frequencies of CXCR5$^+$ CD8$^+$ T-cells in secondary lymphoid organs. Finally, taking advantage of the high proportion of naïve T-cells in huNSG mice, we evaluated the *in vitro* response of splenic T-cells to the follicular profile-polarizing cytokines Transforming Growth Factor (TGF)-β1 and IL-23. After *in vitro* treatment, there was an increase in CXCR5$^+$ CD8$^+$ T-cells, which exhibited high levels of PD-1, CD40 L and low expression of CCR7. Thus, there is a reconstitution of CXCR5$^+$ CD8$^+$ T-cells in huNSG mice, supporting the use of this model for exploring the biology and role of this cell population in healthy and diseased conditions.

1. Introduction

The humanized NOD/SCID/IL-2 receptor γ-chain^{null} (huNSG) mouse model has been widely used for the study of human cells and tissues, as well as the pathogenesis of infections, autoimmunity and cancer, or for pre-clinical studies of drugs and human-cell-based therapies (Koboziev et al., 2015; Shultz et al., 2007). Particularly, this model allows the efficient long-term reconstitution of human T-cell and B-cell populations after engraftment with CD34$^+$ hematopoietic stem cells (HSC) (Ito et al., 2002). Moreover, a partial development of secondary lymphoid organs (SLO) is seen in huNSG mice, with some reports showing lymphoid follicle-like structures in spleen (Ishikawa et al., 2005; Watanabe et al., 2007).

CD4$^+$ T-cells expressing the lymphoid follicle homing chemokine receptor CXCR5 are also reconstituted in some humanized immunodeficient mouse strains bearing human leukocyte antigen (HLA) class II molecules (Allam et al., 2015). However, it is unclear if huNSG mice efficiently reconstitute CXCR5$^+$ CD8$^+$ T-cells. It is also unclear if these cells exhibit a follicular T-cell-like phenotype, such as the expression of Programmed Death (PD)-1, Inducible T-cell costimulatory (ICOS), CD40 L, and the absence of CCR7 (Vinuesa et al., 2016). This CD8$^+$ T-cell subset has attracted attention as it may exert immune surveillance functions in lymphoid follicles (Yu and Ye, 2018). Thus, the reconstitution of CXCR5$^+$ CD8$^+$ T-cells in huNSG mice is relevant for the study of diseases compromising these structures. For instance, during HIV infection, the major viral replication occurs in lymphoid follicles (Folkvord et al., 2005), and follicular CXCR5$^+$ CD4$^+$ T-cells constitute the main viral reservoir (Banga et al., 2016; Perreau et al., 2013). On the other hand, an antiviral function in chronic infections has been demonstrated for follicular CXCR5$^+$ CD8$^+$ T-cells (He and Ye, 2016; Leong et al., 2016; Li et al., 2016; Perdomo-Celis et al., 2017; Rahman et al., 2018). Furthermore, circulating CXCR5-expressing CD8$^+$ T-cells are associated with low-level HIV replication (Perdomo-Celis et al., 2018), making them a possible correlate of protection and a therapeutic tool for targeting HIV reservoirs (Xu et al., 2019). This evidence provides the rationale to boost the CXCR5$^+$ CD8$^+$ T-cell

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response to target HIV reservoirs in lymphoid follicles. This could be accomplished by cell transduction with CXCR5 gene (Ayala et al., 2017), cell stimulation with interleukin (IL)-15 superagonists (Webb et al., 2018), or through priming with Transforming Growth Factor (TGF)-β1 and IL-23, which have been found to promote a follicular CXCR5$^+$ T-cell profile (Mylvaganam et al., 2017; Perdomo-Celis et al., 2019a; Schmitt et al., 2014).

Here, for the first time, we present the characterization of CXCR5$^+$ CD8$^+$ T-cells in huNSG mice. In parallel, we compared the follicular-like profile with that in CXCR5$^+$ CD4 T-cells. In addition, we evaluated the effect of HIV infection on the frequency of these populations. We also assessed the *in vitro* induction of CXCR5$^+$ CD8$^+$ T-cells after treatment with TGF-β1 and IL-23. Overall, our results indicate that CXCR5$^+$ CD8$^+$ T-cells are induced in huNSG mice, with a phenotype and frequency similar to that found in SLO in humans. Moreover, huNSG-derived CD8$^+$ T-cells efficiently respond to TGF-β1 and IL-23, acquiring a follicular-like phenotype. Thus, huNSG mice recapitulate the emergence of CXCR5$^+$ T-cells and are suitable for the study of their dynamics during basal and pathological conditions.

2. Materials and methods

2.1. Generation and HIV infection of humanized mice

All animal care procedures were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland School of Medicine (Protocol number 1018017). Mice were monitored daily for morbidity and mortality, as previously reported (Medina-Moreno et al., 2017). NOD.Cg-*Prkcd*^{scid} *IL2rg*^{tm1Wij}/SzJ (NSG) mice were purchased from The Jackson Laboratory. Two to three-days old NSG mice were gamma-irradiated (100cGy) and engrafted with 1.4 × 10^5 human cord blood-derived CD34$^+$ hematopoietic stem cells (HSCs; Lonza), via intrahepatic injection. Mice were maintained with husbandry conditions and microbiological monitoring practices. At week 12 post-engraftment, mice were checked for human leukocyte reconstitution by flow cytometry, using anti-human CD45-BV421, CD3-APC, CD4-Alexa Fluor 488, and CD8-PerCP-Cy5.5 (Biolegend). Mice with more than 10 % of human CD45$^+$ cells were intraperitoneally infected with 15,000 $TCID_{50}$ units of the CCR5-tropic HIV reference BaL strain. A group of infected mice were treated with combined antiretroviral therapy (ART) consisting of tenofovir disoproxil fumarate (TDF; 200 mg/tablet) plus emtricitabine (FTC; 300 mg/tablet) (Truvada, Gilead Sciences), and raltegravir (RAL; 400 mg/tablet; Isentress, Merck), administered in drinking water. Human-equivalent doses of TDF, FTC and RAL were 61.7, 40.7 and 164 mg/kg/day, respectively, calculated according to *Km* values of 37 and 3 for humans and mice, respectively (Nair and Jacob, 2016). Uninfected mice were included as controls. Mice were serially bled from the retroorbital vein for flow cytometry and viral load analyses and, at week 13 post-infection, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation to obtain secondary lymphoid organs (SLO). Quantification of HIV RNA copy number was done by an in-house real-time RT-PCR, using HIV gag primers SK38/SK39 and SYBR green dyes, as previously reported (Heredia et al., 2015; Medina-Moreno et al., 2017). The assay has a sensitivity of 150 HIV RNA copies/40 μL plasma.

2.2. Flow cytometry

Peripheral blood, spleen and lymph node (LN) mononuclear cells were obtained from mice (LN were not always available). Tissue samples were collected at necropsy and processed immediately in a 70 μm-pore size nylon cell strainer (Corning), followed by mononuclear cell isolation through a Ficoll density gradient (GE Healthcare). Remaining red blood cells were lysed with ACK buffer (Quality Biological Inc.). For phenotypic analyses, cells were incubated for 30 min at room temperature with optimized doses of the following anti-human antibodies: CD3-FITC (clone HIT3a, BD), CD4- BV450 (clone RPA-T4, BD), CD8-Alexa Fluor (AF) 700 (clone OKT8, Thermo Fisher), CXCR5- PerCP Cy5.5 (clone RF8B2, BD), PD-1-BV510 (clone EH12.1, BD), ICOS-AF547 (clone DX29, BD), CCR7-PE (clone 3D12, Thermo Fisher), and CD40 L (clone TRAP1, BD). After incubation, red cells from peripheral blood were lysed with BD FACS Lysing Solution (BD), and all samples were washed twice with FACS buffer (1 % Fetal Bovine Serum in 1X PBS). Finally, cells were fixed with 1 % paraformaldehyde in 1X PBS, and acquired on a FACSAria II cytometer (BD) within an hour of completing the staining. Data were analyzed with the FlowJo Software version 8.7 (Tree Star, Inc.).

2.3. Ex vivo stimulation of splenocytes

For the evaluation of the differentiation profile of splenic T-cells, 1 × 10^6 splenocytes/mL were resuspended in RPMI-1640 supplemented with 10 % fetal bovine serum (both from Gibco), and left resting or stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (at 50 and 500 ng/mL, respectively; both from Sigma-Aldrich) in 96-well U-bottom plates (Costar, Corning, NY). Resting and stimulated cells were incubated for 12 h at 37 °C in 5 % CO_2, all in the presence of 10 mg/mL of Brefeldin A and monensin (both from Thermo Fisher), as well as an optimized dose of anti-human CD107a-APC-H7 (clone H4A3, BD). After culture, cells were collected, washed with FACS buffer and incubated with the following anti-human antibodies: CD3-APC (clone UCHT1, Biolegend), CD8-AF700 (clone OKT8, Thermo Fisher), and CXCR5-PerCP Cy5.5 (clone RF8B2, BD). Next, cells were fixed and permeabilized with Cytofix/Cytoperm and perm wash buffer (BD) and incubated with anti-human granzyme B-FITC (clone GB11, BD), perforin-PE (clone D48, Biolegend), interferon (IFN)-γ-PE Cy7 (clone 4S.B3, BD) and IL-21-BV421 (3A3-N2.1, BD). In a fraction of the animals, cells were also stained with anti-human CD4-BV450 (clone RPA-T4, BD), CD45RA-PE Cy7 (clone L48, Biolegend), and CCR7-PE (clone 3D12, Thermo Fisher). Finally, cells were washed and resuspended in perm wash buffer and acquired on a FACSAria II cytometer.

2.4. In vitro induction of follicular-like CXCR5$^+$ T-cells

The induction of a follicular-like profile in splenic T-cells was documented as previously reported (Perdomo-Celis et al., 2019a). Total splenocytes were independently stimulated at a density of 1 × 10^6 cells/mL in 96-well U-bottom plates with mouse anti-human CD3 (plate-bound for 2 h at 37 °C) and soluble mouse anti-human CD28 (both at 1 μg/mL; clones OKT3 and CD28.2, respectively, Thermo Fisher), in the presence or absence of human recombinant TGF-β1 and IL-23 (at 15 and 75 ng/mL, respectively; Thermo Fisher), and incubated for 5 days at 37 °C in 5 % CO_2. After culture, cell viability was > 85 %, as evaluated by Trypan blue exclusion staining. Then, T-cells were analyzed by flow cytometry to evaluate the expression of CXCR5, PD-1, CD40 L and CCR7, as described above. Here, we report the relative frequency of CXCR5$^+$ T-cells (percentage of the population) with each evaluated condition, as well as the percentage of fold change in the frequency of this subset with cytokine treatments, as follows: % fold change in CXCR5$^+$ T-cells = (anti-CD3/CD28/cytokines treatment minus anti-CD3/CD28 alone condition, divided by anti-CD3/CD28 alone condition) x 100. In a fraction of the experiments, after culture, cells were re-stimulated with PMA/ionomycin for 6 h in the presence of brefeldin A, and subsequently stained for the detection of intracellular IL-21, as described above.

2.5. Histological analysis

To detect follicle structures, spleen and axillary LN fragments were paraffin-embedded and segmented with a microtome (Leica). The resultant fragments (3 − 5 μm of thickness) were placed on charged slides,

deparaffinized, hydrated, and stained with hematoxylin and eosin.

2.6. Statistical analysis

GraphPad Prism software v. 7.0 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Data are presented as medians and interquartile ranges. The Mann–Whitney and Wilcoxon tests were used for comparison of two independent and paired data, respectively. The Kruskal-Wallis and Friedman test were used for comparison of more than two independent and paired data, respectively. The Dunn's test was used for post-hoc analyses. The degree of correlation between variables was determined with the Spearman test. In all the analyses, a value equal to half of the limit of detection of the assay was assigned to samples with non-detectable viral load. In all cases, a P value < 0.05 was considered significant.

3. Results

3.1. CXCR5$^+$ CD8$^+$ T-cells with a follicular-like phenotype are induced in huNSG mice and preferentially confined to SLO

Firstly, the frequency and phenotype of CXCR5$^+$ CD8$^+$ T-cells was evaluated in blood, spleen and LN from huNSG mice. The main observation was the readily generation of this subset in huNSG mice (Fig. 1A), whereas it was not induced in non-humanized NSG mice (data not shown). Consistent with a SLO-homing (Forster et al., 1994; Quigley et al., 2007; Simpson et al., 2010), there was a higher frequency of CXCR5$^+$ CD8$^+$ T-cells in spleen and LN in comparison with blood, with comparable frequencies between both SLO (Fig. 1A and B). This pattern of residency was also observed in CXCR5$^+$ CD4$^+$ T-cells (Fig. 1A and C). CXCR5$^+$ CD8$^+$ T-cells exhibited a follicular-like phenotype (Vinuesa et al., 2016), with a high proportion of CXCR5$^+$ cells expressing PD-1 (Fig. 1A and D), an intermediate proportion of ICOS$^+$ cells (Fig. 1A and E), and a low frequency of CD40L$^+$ cells (Fig. 1A and F). A low proportion of CXCR5$^+$ CD8$^+$ T-cells expressed CCR7 (Fig. 1A and F). Some differences were observed between CXCR5$^+$ CD8$^+$ and CD4$^+$ T-cells. For instance, CXCR5$^+$ CD4$^+$ T-cells exhibited a higher proportion of PD-1$^+$ cells in both SLO, as well as ICOS$^+$ cells in LN (Fig. 1D and E), whereas CXCR5$^+$ CD8$^+$ T-cells had a higher proportion of CD40L$^+$ cells in both SLO, as well as CCR7$^+$ cells in LN (Fig. 1F and G). Moreover, some differences were observed between spleen- and LN-confined cells, with a higher expression of PD-1, but lower expression of CCR7 in CXCR5$^+$ CD4$^+$ T-cells in LN compared with those in spleen (Fig. 1D and G), and a higher expression of CD40 L in spleen-confined CXCR5$^+$ CD8$^+$ T-cells compared with LN cells (Fig. 1F).

To establish if the preferential localization of CXCR5$^+$ T-cells in SLO was associated with the development of follicle structures in huNSG mice, we performed histological analyses in axillary LN and spleen from huNSG mice and compared them with those in non-humanized NSG mice. As shown in Supplementary Fig. 1A and B, follicle-like structures were observed in LN from uninfected huNSG mice, in contrast to non-humanized animals. Similarly, follicle-like structures were observed in spleen from humanized animals (Supplementary Fig. 1C and D). Comparable results were obtained in HIV-infected huNSG mice (data not shown). The follicle-like structures must be confirmed by colocalization studies as reported previously (Ishikawa et al., 2005; Watanabe et al., 2007).

3.2. HIV infection does not affect the frequency of CXCR5$^+$ CD8$^+$ T-cells in SLO from huNSG mice

We evaluated the effect of HIV infection on the magnitude of CXCR5$^+$ CD8$^+$ T-cells in SLO from huNSG mice. First, we determined the response of huNSG mice to the HIV challenge and the effect of ART. Similar to previous reports (Araínga et al., 2016; Medina-Moreno et al., 2017; Satheesan et al., 2018), HIV-infected huNSG mice dramatically increased the viral load between the first and third week post-infection, with stable viral load in the subsequent weeks of monitoring (Supplementary Fig. 2A). Infected mice that received ART exhibited a decrease in the viral load, reaching undetectable levels after two weeks post-treatment initiation (Supplementary Fig. 2A). As expected, no viral load was detected in uninfected mice (Supplementary Fig. 2A). The increase in viral load in untreated HIV-infected mice coincided with the decrease in the CD4:CD8 T-cell ratio in blood (Supplementary Fig. 2B), in accord with the cytopathic effect of HIV on CD4$^+$ T-cells. On the other hand, HIV-infected mice on ART exhibited a recovery in the CD4:CD8 T-cell ratio, reaching levels similar to those in uninfected controls (Supplementary Fig. 2A). Interestingly, when evaluating the frequencies of CXCR5$^+$ CD8$^+$ T-cells in spleen and LN from huNSG mice, we did not observe significant differences in the levels of this subset between HIV-infected and uninfected animals (Fig. 1H). Similar results were obtained for CXCR5$^+$ CD4$^+$ T-cells (Fig. 1I).

3.3. CXCR5$^+$ CD8$^+$ T-cells exhibit a higher expression of IL-21 and greater cytotoxic potential than CXCR5$^-$ cells

We evaluated the functional profile of spleen CXCR5$^+$ CD8$^+$ T-cells after polyclonal stimulation with PMA-ionomycin. First, we focused on IL-21, a cytokine characteristic of follicular T-cells (Vinuesa et al., 2016). Interestingly, after stimulation, CXCR5$^+$ CD8$^+$ T-cells exhibited a higher expression of IL-21 in comparison with CXCR5$^-$ cells (Fig. 2A and B), consistent with a follicular-like profile. Similarly, CXCR5$^+$ CD4$^+$ T-cells had a higher expression of IL-21 in comparison with CXCR5$^-$ cells (Fig. 2A and B). In addition, we evaluated the cytotoxic potential of CXCR5$^+$ CD8$^+$ T-cells by the intracellular expression of the cytotoxic molecules granzyme B and perforin, and we evaluated the degranulation capacity by the surface expression of CD107a; in addition, IFN-γ production was assessed (Fig. 2C). CXCR5$^+$ CD8$^+$ T-cells had a higher co-expression of granzyme B and perforin, as well as higher levels of CD107a compared with CXCR5$^-$ CD8$^+$ T-cells (Fig. 2D), whereas the production of IFN-γ was comparable between both populations (Fig. 2D). These results indicate that CXCR5$^+$ CD8$^+$ T-cells in huNSG mice exhibit some functional characteristics of follicular T-cells, as well as a high cytotoxic potential.

3.4. A low proportion of effector memory T-cells is induced in huNSG mice

Previous reports have shown that CXCR5$^+$ CD8$^+$ T-cells have a memory profile (He and Ye, 2016; Perdomo-Celis et al., 2018; Quigley et al., 2007). Similarly, CXCR5$^+$ CD8$^+$ T-cells in huNSG mice do not express the LN-homing marker CCR7, and express PD-1 (Fig. 1A, D and G), consistent with a memory profile. Considering that there is a poor human T-cell response in huNSG mice product of the lack of HLA molecules that confer antigen-specific T-cell receptor (TCR)-mediated stimulation, we hypothesized that CXCR5$^+$ CD8$^+$ T-cells in huNSG mice originate from xenogeneic reactions with murine major histocompatibility complex (MHC) molecules. Consistent with a low frequency of TCR stimulation, most of splenic CD4$^+$ and CD8$^+$ T-cells from HIV-infected huNSG mice exhibited a CD45RA$^+$ CCR7$^+$ naïve phenotype (Fig. 3A and B) (Sallusto et al., 1999). Nonetheless, we also observed that ~23 % of the CD4$^+$ and ~6 % of the CD8$^+$ T-cells were CD45RA$^-$ CCR7$^-$, a phenotype that characterizes effector memory (EM) cells. Moreover, ~20 % of the CD4$^+$ and ~10 % of the CD8$^+$ T-cells, were CD45RA$^+$ CCR7$^-$ (Fig. 3A and B), a phenotype of terminal effector cells that re-express CD45RA (TEMRA cells). Finally, a low proportion of CD45RA$^-$ CCR7$^+$ T-cells (central memory [CM]) was observed (Fig. 3A and B). To confirm that T-cell naïve and effector phenotypes are reflected in a functional profile, we stimulated splenocytes from HIV-infected huNSG mice with PMA/ionomycin and evaluated the expression of CD107a and granzyme B in CD8$^+$ T-cells. In accord with a human CD8$^+$ T-cell differentiation profile (Opferman et al., 1999; Veiga-Fernandes et al., 2000), naïve CD8$^+$ T-cells had a low to

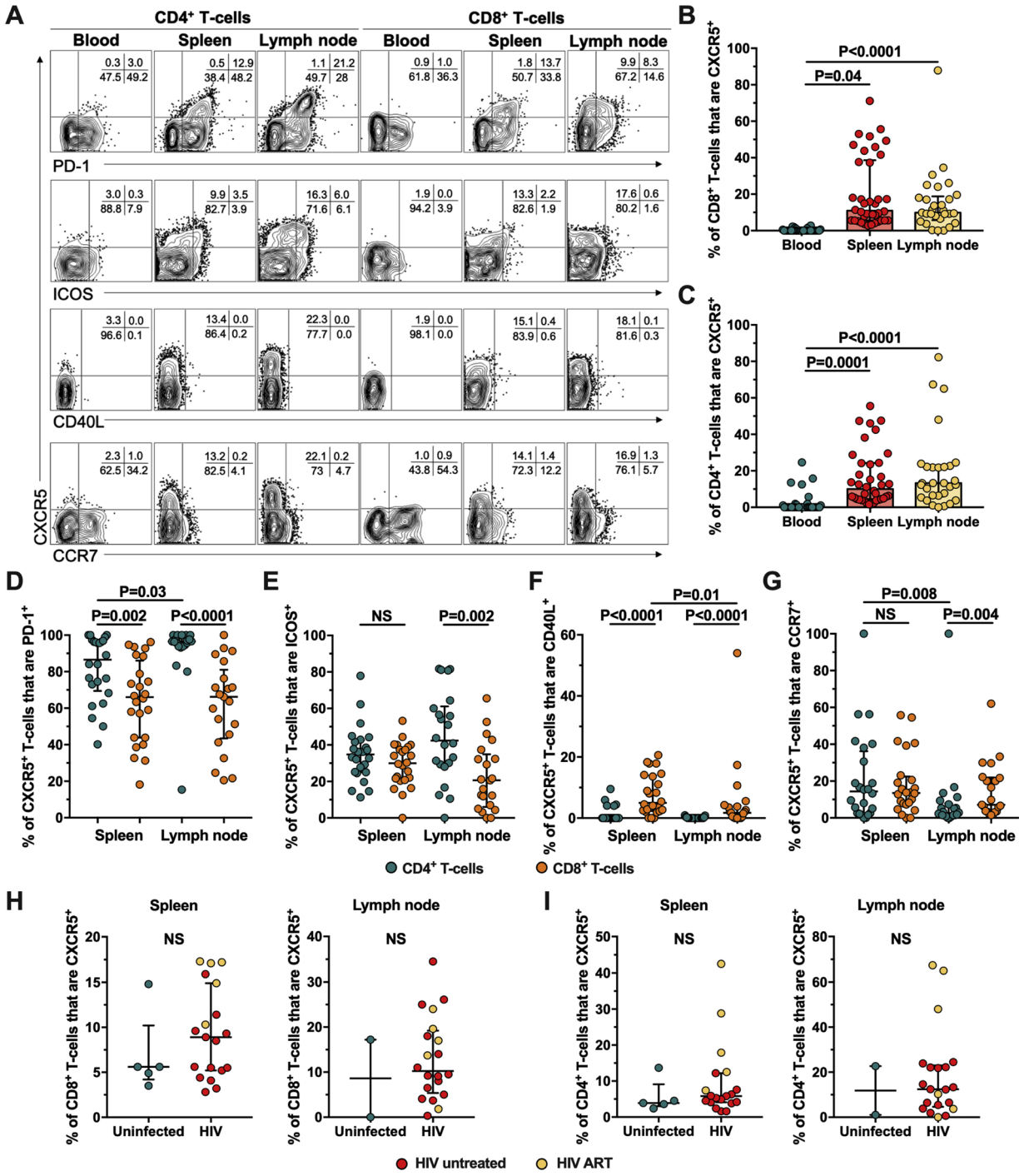

Fig. 1. Generation of CXCR5$^+$ CD8$^+$ T-cells in huNSG mice. A. Representative contour plots from an uninfected huNSG mouse showing the expression of CXCR5, PD-1, ICOS, CD40 L and CCR7 in CD4$^+$ and CD8$^+$ T-cells obtained from blood, spleen and lymph nodes. **B–C.** Frequencies of CXCR5$^+$ CD8$^+$ (**B**) and CD4$^+$ (**C**) T-cells in blood, spleen and lymph nodes from huNSG mice. The P value of the Kruskal-Wallis and Dunn's post-hoc test is shown. **D–G.** Proportion of CXCR5$^+$ CD4$^+$ and CD8$^+$ T-cells that are PD-1$^+$ (**D**), ICOS$^+$ (**E**), CD40L$^+$ (**F**) and CCR7$^+$ (**G**) in spleen and lymph nodes. The P value of the Mann-Whitney test is shown. **H–I.** Frequencies of CXCR5$^+$ CD8$^+$ (**H**) and CD4$^+$ (**I**) T-cells in spleen and lymph nodes from HIV-infected huNSG mice, both untreated and under ART, as well as uninfected controls. The P value of the Mann-Whitney test is shown. NS: Not statistically significant.

undetectable expression of granzyme B and CD107a when unstimulated, but the expression of granzyme B increased after polyclonal stimulation (Fig. 3C and D). On the other hand, EM CD8$^+$ T-cells exhibited a higher expression of granzyme B than naïve cells, and degranulated after polyclonal stimulation (Fig. 3C and D). Thus, a low fraction of T-cells in huNSG mice differentiate to effector cells, possibly through xenogeneic reactions (King et al., 2009), and this process is the probable origin of CXCR5$^+$ CD8$^+$ T-cells in this model.

3.5. TGF-β1 and IL-23 induce follicular-like CXCR5$^+$ CD8$^+$ T-cells

Since follicular CXCR5$^+$ CD4$^+$ T-cells are the main reservoir of HIV (Banga et al., 2016; Perreau et al., 2013), several strategies are being developed to promote antiviral CXCR5$^+$ CD8$^+$ T-cell responses in lymphoid follicles (Xu et al., 2019). One strategy is promoting follicle homing through the induction of CXCR5 in CD8$^+$ T-cells with the use of TGF-β1 and IL-23, in the setting of TCR-mediated stimulation

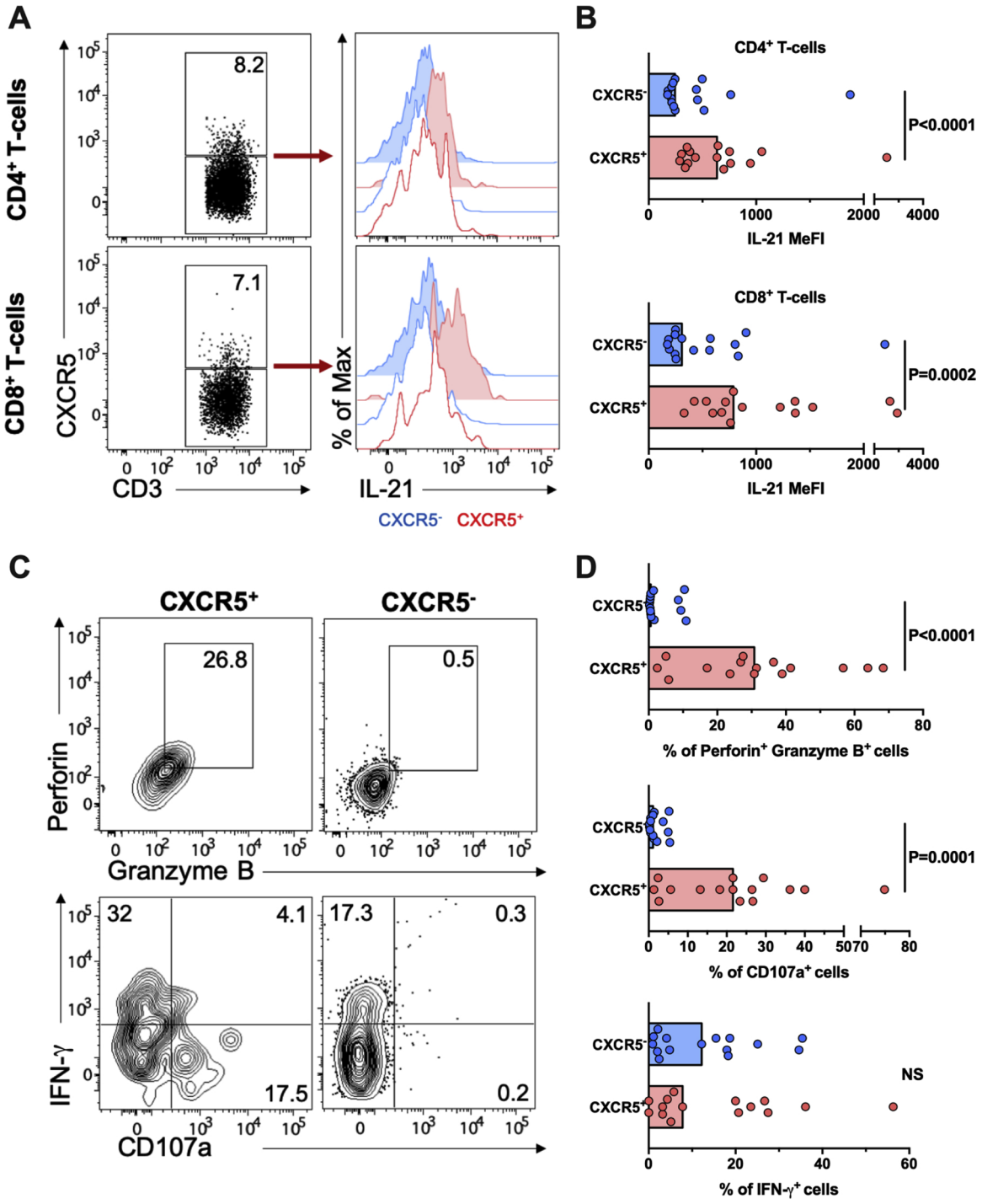

Fig. 2. CXCR5$^+$ CD8$^+$ T-cells express higher levels of IL-21, as well as higher cytotoxic potential, than CXCR5$^-$cells. A. Representative expression (from an uninfected huNSG mouse) of IL-21 in splenic CXCR5$^+$ and CXCR5- CD4$^+$ and CD8$^+$ T-cells after *ex vivo* stimulation with PMA/Ionomycin (filled histograms). Unstimulated cells are shown in open histograms. **B.** Pooled data of the expression of IL-21 (median fluorescence intensity [MeFI]) in splenic CXCR5$^+$ and CXCR5- T-cells after PMA/Ionomycin stimulation. **C.** Representative co-expression of perforin and granzyme B, as well as IFN-γ and CD107a in splenic CXCR5$^+$ and CXCR5- CD8$^+$ T-cells, from an uninfected huNSG mouse, after *ex vivo* stimulation with PMA/Ionomycin. **D.** Pooled data of the frequencies of perforin$^+$ granzyme B$^+$, CD107a$^+$, and IFN-γ$^+$ CXCR5$^+$ and CXCR5- CD8$^+$ T-cells after stimulation. In all the cases, the P value of the Wilcoxon test is shown. NS: Not statistically significant.

(Mylvaganam et al., 2017; Perdomo-Celis et al., 2019a). Thus, we exploited the high proportion of naïve T-cells in spleens from HIV-infected huNSG mice and evaluated the *in vitro* response to TGF-β1 and IL-23, in the setting of TCR-mediated stimulation. As shown in Fig. 4A and B, compared with freshly isolated cells (Fig. 4A and B), a low frequency of CXCR5$^+$ CD8$^+$ T-cells was obtained after 5-days culture with no treatment, that may be due to cell death of a fraction of this population. Consistent with TCR-mediated upregulation and loss of the naïve profile (Ansel et al., 1999; Castle et al., 1993; Rekik et al., 2015; Wherry et al., 2003), the anti-CD3/CD28 treatment increased the frequency of CXCR5$^+$ CD8$^+$ T-cells (Fig. 4A and B), as well as the expression of PD-1 and CD40 L (Fig. 4A, D and E), whereas it decreased the expression of CCR7 (Fig. 4A and F). Interestingly, TGF-β1 and IL-23 further up-regulated the expression of CXCR5 and CD40 L in CD8$^+$ T-

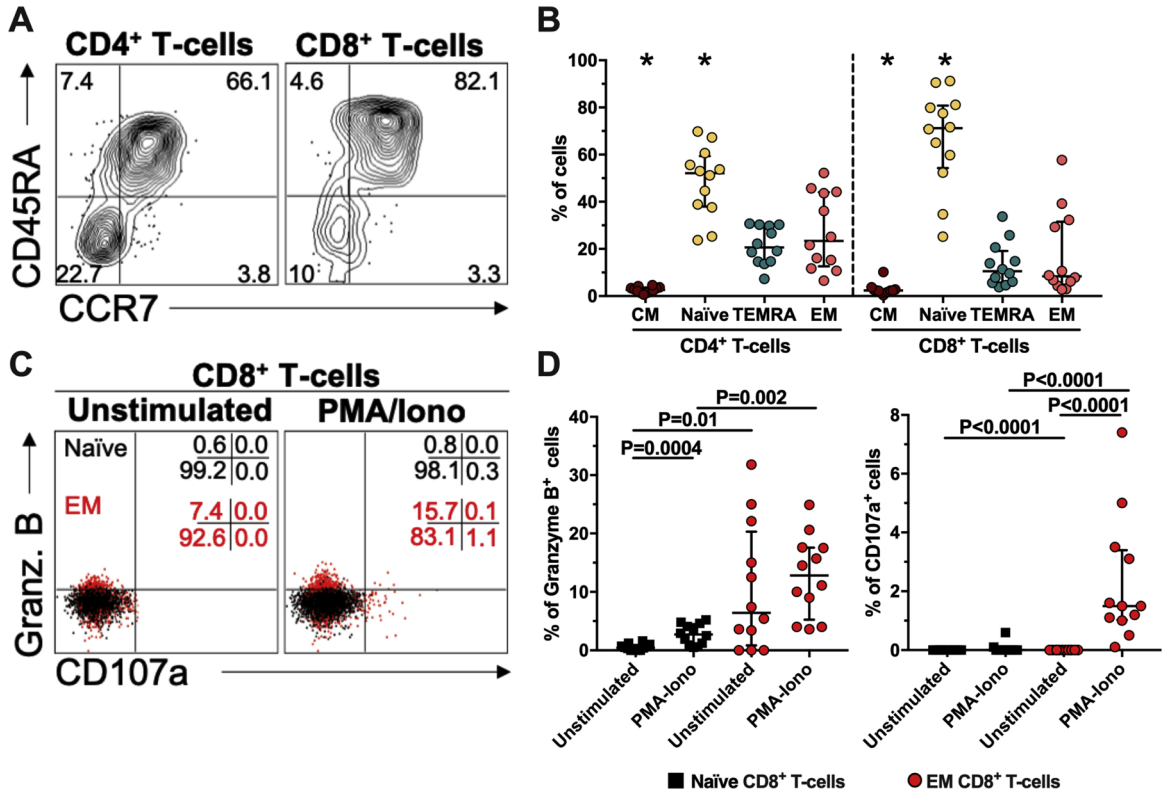

Fig. 3. Differentiation profile of T-cells in huNSG mice. A. Representative contour plots from an HIV-infected huNSG mouse showing the expression of CCR7 and CD45RA in resting splenic CD4$^+$ and CD8$^+$ T-cells. **B.** Pooled data of the frequencies of central memory (CM; CD45RA$^-$ CCR7$^+$), naïve (CD45RA$^+$ CCR7$^+$), terminal effector cells that re-express CD45RA (TEMRA; CD45RA$^+$ CCR7$^-$) and effector memory (EM; CD45RA$^-$ CCR7$^-$) CD4$^+$ and CD8$^+$ T-cells in all the HIV-infected huNSG mice included (n = 12). *P ≤ 0.01 vs. all the other respective T-cell subsets; P value of the Kruskal-Wallis and Dunn's post-hoc test. **C.** Representative dot plots from an HIV-infected huNSG mouse showing the expression of granzyme B and CD107a in resting and PMA/Ionomycin-stimulated splenic naïve (black dots) and EM (red dots) CD8$^+$ T-cells. **D.** Pooled data of the frequencies of granzyme B$^+$ and CD107a$^+$ naïve and EM CD8$^+$ T-cells in all the HIV-infected huNSG mice included (n = 12). The P value of the Mann-Whitney test is shown (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cells (Fig. 4A, B and E), whereas it maintained a high expression of PD-1 but low levels of CCR7 (Fig. 4A, D and F). Overall, similar results were observed for CD4$^+$ T-cells (Fig. 4A, B and D–F).

We also evaluated the % fold change in the expression of CXCR5 in CD4$^+$ and CD8$^+$ T-cells after TGF-β1/IL-23 treatment, in relation to the anti-CD3/CD28 treatment alone. This analysis yielded a median % fold change of ~185 and ~235 in CD4$^+$ and CD8$^+$ T-cells, respectively, without significant differences between both T-cell subsets (Fig. 4C). Of note, the % fold change in the expression of CXCR5 in CD8$^+$ T-cells is at least 3-fold higher than that observed in human blood-derived cells (Perdomo-Celis et al., 2019a), since the pool of circulating naïve CD8$^+$ T-cells in adult humans is lower than that observed in huNSG mice (Thome et al., 2014), and this cell subset is the most readily stimulated by priming/polarizing factors (Croft et al., 1994). We also observed a strong positive correlation between the frequency of naïve T-cells and the % fold change in the expression of CXCR5 in CD4$^+$ and CD8$^+$ T-cells (Fig. 4G), supporting the fact that naïve T-cells are preferentially being stimulated by the anti-CD3/CD28 plus TGF-β1/IL-23 treatment.

Finally, we evaluated the expression of IL-21 by CD8$^+$ T-cells after *in vitro* culture, following re-stimulation with PMA/ionomycin. Interestingly, consistent with the induction of a follicular profile and similar to previous reports (Perdomo-Celis et al., 2019a), there was a trend for a higher expression of IL-21 by total CD8$^+$ T-cells after the treatment with TGF-β1/IL-23 (Fig. 4H). Altogether, these results indicate that TGF-β1 and IL-23 induce follicular-like CXCR5$^+$ CD8$^+$ T-cells.

4. Discussion

Here, we show that SLO-confined CXCR5$^+$ CD8$^+$ T-cells, with a follicular-like profile, are induced in huNSG mice. HIV infection did not affect the frequency of this population. Moreover, the *in vitro* combination of TGF-β1 and IL-23 induced follicular-like CXCR5$^+$ CD8$^+$ T-cells, supporting a role for these cytokines in the polarization of this subset. Overall, these data support that huNSG mice recapitulate some features of human CXCR5$^+$ CD8$^+$ T-cells.

As reported previously in histology studies (Ishikawa et al., 2005; Watanabe et al., 2007), we observed follicle-like structures in LN and spleen from huNSG mice. However, this observation needs further specific staining to phenotype those cells that are clustering together. In addition, by flow cytometry, we observed the generation of CXCR5$^+$ CD8$^+$ T-cells, particularly confined to SLO. Remarkably, their frequencies were similar to those found in non-human primates (NHP) and humans (Ferrando-Martinez et al., 2018; Rahman et al., 2018; Reuter et al., 2017). Also of note, these cells exhibited a follicular-like profile (Vinuesa et al., 2016), as, in addition to the follicle homing marker CXCR5, they expressed PD-1, ICOS and IL-21, and had low expression of CCR7. Moreover, CXCR5$^+$ CD8$^+$ T-cells exhibited a higher cytotoxic potential in comparison with CXCR5$^-$ cells, consistent with an effector memory profile, as previously reported (He et al., 2013; Perdomo-Celis et al., 2018; Quigley et al., 2007).

Contrary to previous human and NHP reports, where an accumulation of follicular CXCR5$^+$ CD8$^+$ T-cells has been evidenced in HIV and Simian Immunodeficiency virus (SIV) infections, respectively (Ferrando-Martinez et al., 2018; Petrovas et al., 2017), we did not

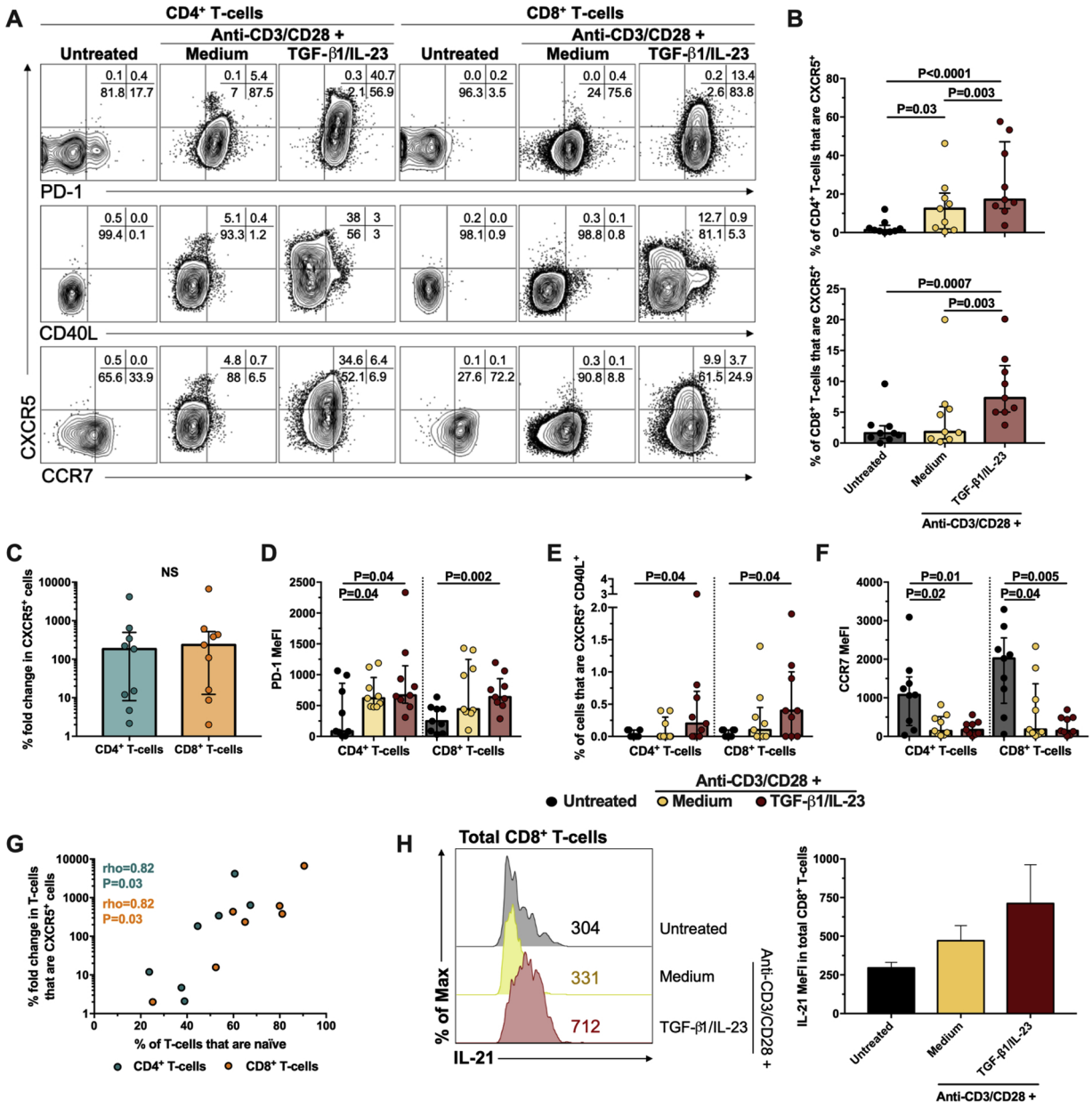

Fig. 4. Induction of follicular-like CXCR5$^+$ CD8$^+$ T-cells by TGF-β1 and IL-23. A. Representative contour plots from an HIV-infected huNSG mouse showing the expression of CXCR5, PD-1, CD40 L and CCR7 in splenic CD4$^+$ and CD8$^+$ T-cells after 5-days culture without stimulation, with anti-CD3/CD28 antibodies alone, or plus TGF-β1 and IL-23. **B.** Frequency of CXCR5$^+$ CD4$^+$ and CD8$^+$ T-cells under the three *in vitro* conditions evaluated. The P value of the Friedman and Dunn's post-hoc test is shown. **C.** % fold change in the frequency of CXCR5$^+$ T-cells after treatment with anti-CD3/CD28 antibodies plus TGF-β1 and IL-23, relative to cells treated with anti-CD3/CD28 alone. NS: Not statistically significant Mann-Whitney test P value. **D.** Median fluorescence intensity (MeFI) of PD-1 in CXCR5$^+$ CD4$^+$ and CD8$^+$ T-cells under the three *in vitro* conditions evaluated. **E.** Frequency of CXCR5$^+$ CD4$^+$ and CD8$^+$ T-cells that are CD40L$^+$ after the *in vitro* treatments. **F.** MeFI of CCR7 in CXCR5$^+$ CD4$^+$ and CD8$^+$ T-cells under the three *in vitro* conditions. In C-F, the P value of the Friedman and Dunn's post-hoc test is shown. **G.** Correlation between the frequency of naïve T-cells (CCR7$^+$ CD45RA$^+$) and the % fold change in the frequency of CXCR5$^+$ T-cells after treatment with anti-CD3/CD28 antibodies plus TGF-β1 and IL-23. The rho and P value of the Spearman test are shown. **H.** Representative expression of IL-21 (MeFI is shown) in splenic CD8$^+$ T-cells after 5-days culture without stimulation, with anti-CD3/CD28 antibodies alone, or plus TGF-β1 and IL-23, followed by re-stimulation with PMA/Ionomycin. The summary of the data is shown on the right (n = 3).

observe differences between HIV-infected and uninfected huNSG mice in the frequencies of SLO-confined CXCR5$^+$ CD8$^+$ T-cells. Comparable frequencies of SLO-confined CXCR5$^+$ CD4$^+$ T-cells between HIV-infected and uninfected huNSG mice were also obtained, contrary to previous human and humanized mice studies, where an accumulation of this subset has also been reported (Allam et al., 2015; Lindqvist et al., 2012). In the case of follicular CXCR5$^+$ CD8$^+$ T-cells, a local inflammatory environment and increased levels of CXCL13 (ligand of CXCR5) are the cause of their increase in follicles during HIV/SIV infection (Ferrando-Martinez et al., 2018; Petrovas et al., 2017). On the other hand, follicular CXCR5$^+$ CD4$^+$ T-cells are an important target of HIV (Banga et al., 2016; Perreau et al., 2013), and the presence of high levels of virions captured by follicular dendritic cells (Spiegel et al., 1992), among other factors, are responsible for a heightened viral burden in lymphoid follicles (reviewed in Leong et al., 2017). Overall, the conditions that determine follicular CXCR5$^+$ CD4$^+$ T-cells as major targets of HIV, as well as the local inflammatory environment that induces the follicle migration of CXCR5$^+$ CD8$^+$ T-cells, are apparently

not present in huNSG mice, as they do not develop follicle structures and there is poor reconstitution of dendritic cells, critical for the development of follicles (Cyster et al., 2000). These circumstances might be related with the unchanged frequencies of CXCR5$^+$ CD8$^+$ and CD4$^+$ T-cells in HIV-infected huNSG mice.

Considering that NSG mice lack HLA molecules in the thymus that are necessary for the positive selection of human T-cells, a weak cellular immune response is expected. Nonetheless, we show that CXCR5$^+$ CD8$^+$T-cells are readily induced in huNSG mice. The induction of CXCR5$^+$ CD8$^+$ T-cells is not limited to huNSG mice, as we have shown reconstitution of these populations in humanized NOD/Shi-*scid*-IL2rγ^{null} (NOG) mice bearing the human genes for IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (NOG-EXL mice) (Perdomo-Celis et al., 2019b). Since reconstituted T-cells in huNSG mice are not expected to receive antigen-specific signals through their TCR, CXCR5$^+$ CD8$^+$ T-cells in huNSG mice are probably induced by a xenogeneic reaction with murine MHC molecules (King et al., 2009). Indeed, the human T-cell-murine MHC interaction in humanized mice promotes an effector memory profile and production of IFN-γ (Ali et al., 2012; Covassin et al., 2011), similar to our results. Further studies evaluating the induction of CXCR5$^+$ CD8$^+$ T-cells in huNSG mice by stimulation of reconstituted naïve T-cells with murine antigen-presenting cells could elucidate this issue.

In order to promote antiviral immune responses in lymphoid follicles, which could be useful for targeting HIV reservoirs, different groups have developed strategies focusing on enhancing the expression of CXCR5 in T-cells (Xu et al., 2019). For instance, in SIV infection models, the transduction of CD8$^+$ T-cells with CXCR5 promoted their migration into follicles proximal to SIV-infected cells (Ayala et al., 2017). CD8$^+$ T-cells bearing SIV-specific chimeric antigen receptors in conjunction with CXCR5 also migrated into lymphoid follicles (Haran et al., 2018). Moreover, an IL-15 superagonist upregulates CXCR5 in SIV-specific CD8$^+$ T-cells, allowing them to enter lymphoid follicles and exert antiviral control (Webb et al., 2018). These follicular CXCR5$^+$ CD8$^+$ T-cells could readily eliminate HIV-infected cells (Petrovas et al., 2017), promoting reservoir purging. In addition, *in vitro* treatment of T-cells with TGF-β1/IL-23 induces follicular-like CXCR5$^+$ T-cells, with upregulation of PD-1, ICOS, CD40 L, IL-21 and the transcription factor B-cell lymphoma (BCL)-6, and down-regulation of B-lymphocyte-induced maturation protein-1 (BLIMP-1) (Mylvaganam et al., 2017; Perdomo-Celis et al., 2019a; Schmitt et al., 2014). Accordingly, we observed that these cytokines, in the setting of TCR stimulation, induced follicular-like CXCR5$^+$ CD8$^+$ T-cells from huNSG mouse spleens. Remarkably, our results indicate that follicular-like CXCR5$^+$ T-cells were mostly derived from naïve cells. Considering that HIV-infected patients have decreased frequencies of naïve T-cells (Chen et al., 2001; Tanko et al., 2018), these results are in agreement with the limited response to TGF-β1/IL-23 of CD8$^+$ T-cells from HIV-infected patients (Perdomo-Celis et al., 2019a). Altogether, our results indicate that TGF-β1 and IL-23 *in vitro,* induce follicular-like CXCR5$^+$ CD8$^+$ T-cells in cells derived from huNSG mice, and together with the generation of these populations after HSC engraftment, support the use of this model for the study of CXCR5$^+$ CD8$^+$ T-cells and potential strategies for boosting their antiviral response. These analyses could be improved, for instance, with the use of huNSG mice with transgenic expression of HLA molecules, as well as human TGF-β1 and IL-23. Importantly, since TGF-β1 and IL-23 have systemic pleiotropic effects, and particularly since TGF-β1 promotes tissue fibrosis that is associated with AIDS progression (Zeng et al., 2011), the *in vivo* administration of TGF-β1 and IL-23 to HIV-infected patients as a therapeutic approach for boosting CXCR5$^+$ CD8$^+$ T-cells responses is not appropriate. Instead, the *in vitro* induction of follicular-like CXCR5$^+$ CD8$^+$ T-cells could be a potential strategy for cell-based therapeutics, such as adoptive transfer into HIV patients to enhance antiviral responses in lymphoid follicles.

Another potential antiviral role of CXCR5$^+$ CD8$^+$ T-cells is the stimulation of anti-HIV B-cell responses. In fact, CXCR5$^+$ CD8$^+$ T-cells exhibit some phenotypic, transcriptional and functional similarities with CXCR5$^+$ CD4$^+$ T-cells (Vinuesa et al., 2016), suggesting that they can stimulate B-cells. Indeed, an increase in the survival and IgG production by B-cells from human tonsils has been observed when they are cocultured with tonsil CXCR5$^+$ CD8$^+$ T-cells (Quigley et al., 2007). Another report also showed that the addition of human blood-sorted CXCR5$^+$ CD8$^+$ T-cells to B-cells-CXCR5$^+$ CD4$^+$ T-cells cocultures increased the secretion of IgG and IgA (Jiang et al., 2017). Moreover, human tonsil-derived CXCR5$^+$ CD8$^+$ T-cells promoted the secretion of IgG, IgM and IgA by purified B-cells in a mechanism mediated by IL-21 and CD40 L (Shen et al., 2018). However, the role *in vivo* of CXCR5$^+$ CD8$^+$ T-cells on the promotion of B-cell antibody responses during HIV infection is unknown.

There are some limitations in using huNSG mice for the study of CXCR5$^+$ CD8$^+$ T-cells and effector functions. As mentioned, reconstituted human T-cells do not receive thymic education and TCR-mediated signals, restraining the study of antigen-specific responses. Moreover, there is a poor development of myeloid populations, B-cells and lymphoid follicle structures, which also limit the evaluation of the potential effect of CXCR5$^+$ CD8$^+$ T-cells on humoral responses (Seung and Tager, 2013). The use of transgenic or bone marrow-liver-thymus (BLT) mouse strains could overcome these limitations. Further, the origin of CXCR5$^+$ CD8$^+$ T-cells in huNSG mice remains to be defined, as well as whether they are transcriptionally and functionally comparable to those found in humans.

Author contributions

FP-C wrote initial project, performed experiments, analyzed the results and wrote the first manuscript draft; SM-M performed experiments and reviewed and edited the manuscript; HD, JB, and SK reviewed and edited the manuscript; NAT and MTR wrote initial project, reviewed and edited the manuscript; JCZ wrote initial project, performed experiments, performed project supervision and administration, reviewed and edited the manuscript.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.11.020.

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