A Human Lin− CD123+ CD127low Population Endowed with ILC Features and Migratory Capabilities Contributes to Immunopathological Hallmarks of Psoriasis

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Innate lymphoid cells (ILC) are members of a heterogeneous family with a lymphoid origin that mimics the Th helper (Th) cytokine profile. ILC are involved in early effector cytokine-mediated responses during infections in peripheral tissues. ILC also play an important role in chronic skin inflammatory diseases, including psoriasis. Although classical ILC express CD127, it has been recently reported that the presence of non-classical CD127− ILC populations and an early ILC precursor (EILP) CD127low. ILC development has predominately been investigated in mouse models. However, in humans, different transcription factors have been described for ILC identification. NFIL3 (nuclear factor, IL-3 regulated) is crucial for ILC development in response to IL-7. CD123 (IL-3Rα) is usually used to exclude basophils during ILC identification, however, it is unknown if in response to IL-3, NFIL3 could be relevant to induce ILC features in Lin− CD123+ populations in addition, is also unknown whether peripheral blood (PB) population with ILC features may have skin-homing potential to participate in skin inflammatory chronic diseases. Here, we report a Lin− CD123+ CD127low CD7+ CLA+ population that share some phenotypic properties with basophils, but expresses several transcription factors that are characteristic of ILC.

Abbreviations: AhR, aryl hydrocarbon receptor; BCR, B cell receptor; BDCA, blood dendritic cell antigen; cDC, conventional dendritic cells; CLA, cutaneous lymphocyte antigen; CLP, common lymphoid precursor; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells or prostaglandin D2 receptor 2; CS, control skin; GATA-3, trans-acting T-cell-specific transcription factor; HD, healthy donors; HLA-DR, human leukocyte antigen-antigen D related; HUVEC, human umbilical vein endothelial cells; ILC, innate lymphoid cells; NCR, natural cytotoxicity receptors; NFIL3, nuclear factor, IL-3 regulated; NK, natural killer; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; pDCs, plasmacytoid dendritic cells; PLZF, promyelocytic leukemia zinc finger; PMA, phorbol 12-myristate 13-acetate; ROR-γt, RAR-related orphan receptor gamma; SDF-1, stromal cell-derived factor-1; T-bet, T-box transcription factor; TCR, T cell receptor; TCF-1, T cell factor-1; Th, Th helper; TOX, thymocyte selection-associated high-mobility group box protein.
INTRODUCTION

Innate lymphoid cells (ILC) have been defined as a heterogeneous family derived from a CD7+ common lymphoid precursor (CLP) (1–3). During the previous decade, several ILC populations that participate in the defense against pathogens and inflammatory diseases have been described mainly in mice (4, 5). The identification of ILC populations in humans, as well as their role in disease pathogenesis, comprises a topic of extensive investigation.

Several groups have used distinct criteria and markers for ILC identification. ILC have a classical lymphoid morphology; they lack T cell receptor or BCR expression and are considered lineage negative (Lin-) cells. In humans, the most common lineage markers include CD3, CD19 (for T and B cells), CD14 (monocytes), and CD11c, as well as blood dendritic cell antigen (BDCA)-1, -2 (dendritic cells). CD123 expression has been used to exclude plasmacytoid dendritic cells (pDCs) and basophils, while FcεR for basophils and mast cells (6, 7).

Classical ILC in human peripheral blood (PB) express CD127. However, a non-classical category of ILC CD127− (8) as well as an early ILC precursor (EILP) CD127low (9) has been recently reported. In addition to CD127, other markers have been used in human PB for ILC identification, ILC express CD132 (γ common chain), which is crucial for development, as well as CD90 and CD161 as ILC markers (2). The expression of α4β7 integrin has been reported in ILC precursors in mice (10–12). In addition to surface markers, several transcription factors have been used for ILC identification. The transcription factor inhibitor of DNA binding 2 (Id2), NFIL3, promyelocytic leukemia zinc finger (PLZF), for ILC commitment such as inhibitor of DNA binding 2 (Id2), NFIL3, promyelocytic leukemia zinc finger (PLZF), thymocyte selection-associated high-mobility group box protein (TOX), and T cell factor-1 (TCF-1). In addition, this population expresses different ILC markers: CD132, CD90, CD161, α4 integrin, c-Kit, CRTH2, AhR, and IL-23R. IL-3 prevents apoptosis and increases their NFIL3, TOX, and PLZF expression. In PB, the CD123+ CD127low population is predominantly a conspicuous population that expresses T-bet and RORγt. The Lin− CD123+ CD127low population in PB has a limited Th type cytokine expression and highly expresses IL-8. The Lin− CD123+ CD127low population expresses skin-homing receptors (cutaneous lymphocyte antigen and CXCR4) and transmigrates through endothelial cells in response to SDF-1. An equivalent Lin− CD123low population was identified in control skin, which shows a broader phenotypic diversity and cytokine production, including IL-22 and IL-17. Remarkably, the CD123low population in the lesion and non-lesion skin of psoriasis patients expresses IL-17 and IL-22. Our findings suggest the identification of an alternative Lin− CD123+ CD127low population with ILC features endowed with migratory capabilities that might contribute to immunopathological hallmarks of psoriasis.

Keywords: innate lymphoid cells, psoriasis, IL-3Rx, IL-17, SDF-1, CXCR4 axis, skin inflammation
against pathogens (28–31). Recently, a regulatory role for ILC populations have been reported (32). Therefore, in humans, there is increasing evidence that ILC play a role in several pathologies, such as allergies and chronic inflammatory skin disorders (33), including psoriasis (34, 35). Interestingly, the proportions of the different subsets (ILC1, ILC2, and ILC3) among tissues appear to be different, and it also appears that the local microenvironment may influence the "specialized" functions of ILC (36, 37). It has been proposed that ILC in PB may represent a reservoir of ILC in which their functional features may be distinct from peripheral tissues (7, 24, 38). Nevertheless, the mechanisms that underlie the migration of ILC into different tissues under steady state or inflammatory conditions are in the early stages of investigation. In particular, for skin migration, it has been reported that in PB, ILC2 and ILC3 express cutaneous lymphocyte antigen (CLA) (39, 40), which is the main assumed mechanism of ILC skin tropism under steady-state conditions; however, additional migration mechanisms under inflammatory conditions have not been established to date.

In the skin, one of the main human pathologies in which the participation of ILC has been investigated is psoriasis. It has been described that blood and skin samples from patients the participation of ILC has been investigated is psoriasis. It has been proposed that ILC in PB may represent a reservoir of ILC in which their functional features may be distinct from peripheral tissues (7, 24, 38). Nevertheless, the mechanisms that underlie the migration of ILC into different tissues under steady state or inflammatory conditions are in the early stages of investigation. In particular, for skin migration, it has been reported that in PB, ILC2 and ILC3 express cutaneous lymphocyte antigen (CLA) (39, 40), which is the main assumed mechanism of ILC skin tropism under steady-state conditions; however, additional migration mechanisms under inflammatory conditions have not been established to date.

Here, we identified a Lin− CD123+ CD127low population in the PB of healthy donors (HD) that express several ILC features and in which IL-3 appears to be essential for their maintenance and identity. Interestingly, this Lin− CD123+ CD127low population highly expresses CLA and exhibits migratory potential in response to SDF-1. Remarkably, a similar Lin− CD123low population was identified in control skin (CS) and importantly in psoriasis skin (PS) biopsies with the capability to express IL-22 and IL-17. These findings suggest that this population with ILC features may contribute to the immunopathological features of psoriasis.

**MATERIALS AND METHODS**

**Blood Sample Collection**

Buffy coats of HD were obtained from the Blood Bank from the “Hospital Infantil de México: Federico Gómez.” Peripheral blood mononuclear cells (PBMCs) were isolated with Lymphoprep (Axis-Shield, Oslo, Norway) fromuffy coats.

**Skin Biopsies from CS and Psoriasis Patients**

Control skin was obtained from remnant skin following plastic or abdominal surgeries that was free from dermatologic pathologies from the “Hospital de Especialidades Dr. Bernardo Sepulveda CMN Siglo XXI.” Patients were recruited from the dermatology clinic of the Centro Dermatológico Dr. Ladislao de la Pascua. Fifteen patients who fulfilled the diagnostic criteria for psoriasis in plaque were included in this study prior to treatment initiation. Patient biopsies were obtained with a 5–6 mm punch.

**Skin Cell Collection**

Skin samples were placed overnight in RPMI medium and dispase II (Grade II protease, Roche, Switzerland). The dermis was then mechanically separated from the epidermis. Dermal cells were obtained by allowing migration from dermal segments placed in culture in RPMI medium for 7 days. The collected cells were used for activation experiments, and the cell supernatants were used as chemoattractant stimuli in migration assays.

**Flow Cytometry Analysis and Sorting**

In order to block Fc receptors, PBMCs were incubated with an in-house-made buffer containing 2% horse serum. Cells were stained with a cocktail of antibodies (complete list included in Supplementary Material). Fixation was performed using 4% paraformaldehyde (PFA). All antibodies were isotype-matched with their respective fluorophore. Intracellular assessment of cytokines and transcriptional factors were performed using Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA) or the Factor Fixation and Permeabilization Buffer Set (Biolegend, San Diego, CA, USA), respectively. Cells were incubated with antibodies for 30 min at room temperature. Before cell fixation, Hoechst 33342 staining assessed cell viability during assays. The samples were acquired using a FACS Canto (BD Biosciences, San Jose, CA, USA) and were analyzed with Flowjo software (Tree Star). For cell isolation, PBMCs were stained with PE-conjugated lineage marker antibodies. Lineage+ cells (CD3+, CD14+, CD19+, CD94+, and HLA-DR+) were depleted using anti-PE Microbeads and LD columns (Milenyi-Biotec, BG, Germany). The cells were sorted from the lymphoid region and according to CD123 and CD127 expression using a FACS Aria II (BD Biosciences, San Jose, CA, USA).

**Heat Map Construction**

The median fluorescence intensities (MFs) were determined for each cell surface marker and each subpopulation; the minimum value reported (gray) corresponds to the isotype control (MF), and the maximum value reported (red) corresponds to the cell population with the highest MF value.

**Imaging Flow Cytometry**

The morphology of total pre-enriched Lin− CD123+ cells was evaluated in PBMCs previous to depletion of Lineage+ cells (as described above) and stained with anti-CD123 and the nuclear dye DAPI (Thermo Fisher Scientific, MA, USA). Cells were acquired using the Amnis ImageStream Mark II and analyzed by the IDEAS® software (Merck-Millipore, MA, USA).

**Quantitative RT-PCR**

Total RNA was extracted from a pool of three different cell donors using RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, MA, USA), according
to the manufacturer’s instructions. Quantitative gene expression for human IL7Rα or for the housekeeping human GAPDH gene was performed using Maxima Syber Green qPCR Master Mix (Thermo Fisher Scientific, MA, USA) and a Rotorgene Real Time PCR System (Qiagen, Hilden, Germany). Used primers: IL7Rα-Forward: 5’ AGG ATG AAA ACA AAT GGA CGC A 3’, IL7Rα-Reverse: 5’ CCT TTA AAA TAG TGA TCA GGG ATG G 3’. Size of cDNA product: 238 bp.

Incomplete D_H−J_H Rearrangements Analysis
Genomic DNA was extracted from sorted cell populations using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The identification of incomplete D_H–J_H rearrangements was performed using the BIOMED-2 primer sets as described (42). Briefly, two independent PCR reactions per DNA sample were set: a multiplexed PCR reaction combining a single J_H consensus primer and six D_H primers (Tube D) corresponding to six of the seven D_H segment families. The second reaction contained the same J_H primer and a single D_H/7 primer (Tube E). Each reaction product was subjected to capillary electrophoresis using the Agilent DNA 1000 chip in the Agilent 2100 Bioanalyzer system.

Cell Activation
Peripheral blood mononuclear cells or skin cells were stimulated using the cell stimulation cocktail and the transport protein inhibition cocktail (for the last 6 h of culture) (eBioscience-Affymetrix, Santa Clara, CA, USA) to assess the production of cytokines via intracellular staining. When indicated, IL-3 was added to the culture for 18 h to evaluate the phenotype and expression of transcriptional factors. Furthermore, ILC1, ILC2, and ILC3 cocktails (Supplementary Material) were added to the culture for 18 h to determine the expression of intracellular cytokines; IL-3 and IL-7 were included in all cocktails.

IgE Crosslinking Assay
Peripheral blood mononuclear cells were incubated with 1 µg/mL of human IgE (Merck-Millipore, MA, USA) during 2 h, washed, and incubated with 2 µg/mL of anti-human IgE (BD Biosciences, San Jose, CA, USA) during 30 min; the activation of cells was assessed by the expression of phenotypic markers at the end of incubation.

Immunofluorescence
Goat anti-Id2 (R&D Systems, Minneapolis, MN, USA), Biotin anti-goat IgG, Isotype control, AlexaFluor 488 Streptavidin (Jackson-Immunoresearch, West Grove, CA, USA) and Vectashield-DAPI (Vector-laboratories, Burlingame, CA, USA) were used to stain sorted populations. Images were acquired using a Leica TCS SP8x Confocal Microscope (Wetzlar, Germany) and were analyzed with Leica software.

Cell Viability Assay
Isolated Lin− CD123+ CD127low cells were cultured in the presence or absence of IL-3 (PeproTech, NJ, USA) for 3 days. Cells were collected and stained using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions. Samples were acquired using a FACS Canto (BD Biosciences, San Jose, CA, USA) and were analyzed with Flowjo software (Tree Star).

Cytokine Quantification
Isolated Lin− CD123+ CD127low from the lymphoid region or Lin− CD127+ cells were cultured in the presence or absence of IL-3 (PeproTech, NJ, USA) for 18 h ± stimulation cocktail (eBioscience-Affymetrix, Santa Clara, CA, USA) during the last 6 h of culture. The supernatants were collected and stored (−80°C) until analysis. The quantification was performed using a personalized (Supplementary Material) Magnetic Luminex Screening Assay (R&D Systems, Minneapolis, MN, USA). Sample acquisition and analysis were performed in a MagPix instrument (Luminex, TX, USA).

Migration Assay in Transwell System
Using a transwell system (Corning, NY, USA), migration in response to stimuli, SDF-1 (R&D systems, Minneapolis, MN, USA) or supernatants from CS or PS cell cultures was assessed after 3 h. Migration was also assessed in the presence of blocking anti-CXCR4 (Biolegend, San Diego, CA, USA). The transmigration assays using endothelial cells, the upper chamber of the transwell was covered with attachment factor (Gibco, Thermo Scientific, MA, USA) during 1 h/37°. Human umbilical vein endothelial cells were cultured for 1–2 days (with supplemented EGM-2 medium, Lonza, Switzerland) in the previous covered well to promote monolayer formation. The monolayer was subsequently washed with PBS 1x and used for the migration assay. 2 × 10^6 PBMCs per milliliter were placed in the upper chamber in the presence or absence of SDF-1 as stimuli to assess migration after 4 h. Cells from the upper and lower chambers were counted using a microscope and were analyzed by flow cytometry.

Statistical Analysis
Statistical analysis was performed using the GraphPad Prism 5.0 (La Jolla, CA, USA) software. The non-parametric Mann–Whitney U test was used to calculate the statistical significance between the groups. All p values less than 0.05 were considered statistically significant.

RESULTS
A Human Lin− CD123+ CD127low Cell Population with Lymphoid and Basophil Features in PB
To determine the presence of Lin− CD123+ (IL-3Rα) population in the PB of HD, cells with a lymphoid morphology and lineage negative (Lin−) for T cells (CD3−), B cells (CD19−), monocytes (CD14−), NK cells (CD56−), and dendritic cells (HLA-DR+) were evaluated. Two distinct Lin− cell populations were identified: the classical CD127+ population and a CD123+ with low expression of CD127 by both, flow cytometry and PCR (Figures 1A,B). The cell frequency of the CD123+ CD127low population is approximately 0.7–1.4%, while the CD127+ population is...
approximately 0.09–0.15% of the total PBMC cells. The CD123+ CD127low population is about 8–10 times more frequent than the CD127+ population (Figure 1A). As indicated in Figure 1C, both the Lin− CD123+ CD127low and CD127+ populations are negative for CD34, suggesting that they are not primitive precursor cells, as well as for T cells (TCRαβ), granulocytes (IL-5R, CD177, and CD66) and NK, monocytes, and neutrophil (CD16). The Lin− CD123+ CD127low was also negative for conventional dendritic cells (BDCA-1, BDCA-3, and CD11c) and pDCs (BDCA-2, BDCA-4, and HLA-DR) (Figure 1D; Figure S1 in Supplementary Material). As indicated in Figure S1 in Supplementary Material, the Lin− CD123+ CD127low population was absent when anti-FcεR was included in the lineage cocktail. Therefore, in addition to CD123, we evaluated its morphology and expression of basophil markers, including FcεR, CCR3, CD203c, and an antigen expressed in the secretory granules of human basophils that is recognized by the monoclonal antibody 2D7 (43). Remarkably, imaging flow cytometry assay identified two populations of total pre-enriched Lin− CD123+. As observed in Figure 1E, there is a mixture of lymphocyte cells with not segmented nuclei (no lobes), and cells with a classical basophil morphology (lobed nuclei). In addition, two cell populations by two different gating strategies were identified when Lin− CD123+ and then FSC vs. SSC were analyzed (Figure 1F) upon FSC
vs. SSC and then Lin− vs. CD123+ analysis (Figure 1G). These two cell populations were further defined as FSCint SSCint (1) and FSCint SSCint (2) with a frequency of 0.3–1.1 and 0.7–1.3%, within total PBMCs, respectively. Both populations showed similar expression of FceR and CCR3. However, the expression of CD203c and the antigen identified by the 2D7 mAb are substantially lower in the CD123− CD127low population from the lymphoid region, consistent with its morphology (Figure 1H). To evaluate the possible lymphoid origin of the Lin− CD123+ CD127low population, we investigated the presence of incomplete D(N−JH) rearrangements as a molecular fingerprint of early lymphoid precursors. As shown in Figures S2 and S3 in Supplementary Material, we could not find significant D(N−JH) rearrangements in the CD127+ classical ILC or in the Lin− CD123+ CD127low population when compared with B-lymphocytes and acute B cell leukemia cell line NAL-M6. However, the expression of CD7 distinguished the lymphoid-related CD123+ cells from the CD123+ FSCint SSCint population and monocytes (Figures 1I,J). These findings indicate the presence of two Lin−CD123+ populations in PB and suggest that CD123+ CD127low cells express a mixture of basophil and lymphoid features.

A Human Lin− CD123+ CD127low CLA+ Population with ILC Features in PB
Several ILC markers were investigated in the Lin− CD123+ CD127low within the lymphoid region. Importantly, such population expressed high levels of CD132 and CD90, compared to the Lin− CD127+ population, but low levels of CD161 (Figure 2A). In addition, both populations were similar in the low expression of α4 integrin that has been reported in ILC precursors. Higher levels of c-Kit and CCRTH2, AhR and IL23R were observed in the Lin− CD123+ CD127low when compared to the Lin− CD127+ counterpart, but both populations were NKp44−. Although both populations displayed similar amounts of CCR6, the Lin− CD123+ CD127low population exhibited a remarkable expression of CXCR4 and of the CLA (Figure 2A).

In addition, the transcriptional factor Id2, which is crucial for ILC commitment, was confirmed in these cells. Notably, the Lin− CD123+ CD127low and CD127+ populations express Id2 predominantly within nuclei compared to the isotype control, although the staining pattern was distinct (Figures 2B–F).

Identity and Maintenance of the ILC Features Are Mediated by IL-3
Next, the effect of IL-3 was assessed in the Lin− CD123+ CD127low population. Sorted Lin− CD123+ CD127low from the lymphoid region cells were cultured in the presence of IL-3; after 3 days, IL-3 enabled the survival of the cells, as more than 90% of the cells cultured without IL-3 died via apoptosis (Figure 3A). In addition to Id2 NFIL3, PLZF, TOX, and TCF-1 have been reported as crucial in ILC development (15–17). Notably, the Lin− CD123+ CD127low population expressed similar levels of NFIL3 compared with the classic CD127+ ILC; however, the CD127+ population exhibited a higher expression of PLZF, TOX, and TCF-1. Remarkably, IL-3 was capable of increasing the expression of NFIL3, PLZF, and TOX in the Lin− CD123+ CD127low population in contrast to the CD127+ population (Figures 3B–E). These findings suggest that IL-3 has two main roles in the CD123+ CD127low population: maintenance of cell survival and the upregulation of crucial transcriptional factors related to ILC identity.

The Lin− CD123+ CD127low Population Is Primarily a Conspicuous Population That Expresses T-bet and RORγt
Innate lymphoid cell populations are classified by phenotype in ILC1, ILC2, and ILC3 (44). In this regard, we determined that the majority of cells within the Lin− CD123+ CD127low population displayed a phenotype similar to ILC2, due to expression of CD161−, c-Kit, and CCRTH2, although expression of CCRTH2 may resemble a basophil-like phenotype. In contrast, a limited number of cells with an ILC1 (CRTH2−, c-Kit+) or ILC3 (CRTH2−, c-Kit+, NKp44+) phenotype were identified (Figure 4A). In contrast, in the CD127+ ILC, we identified two populations by the expression of CD161, and ILC1, ILC2, and ILC3 (NKp44) populations were identified (Figure 4B). Remarkably, the Lin− CD123+ CD127low population had similar expressions of T-bet and RORγt compared with the CD127+ population; however, there was only a slight expression of GATA-3 (Figure 4C). The small amount of diversity and the expression of T-bet, RORγt, or GATA were not affected in the Lin− CD123+ CD127low population by IL-3 culture (data not shown). These findings indicated that the Lin− CD123+ CD127low population in PB is primarily a conspicuous population with minority phenotypic diversity that expresses T-bet and RORγt, which suggests that these transcription factors may be involved in their function.

Steady-State Peripheral Lin− CD123+ CD127low Population Expresses Limited Th Type Cytokine Variety
The ILC1, 2, and 3 populations have been described as innate analogs to Th lymphocytes by their capacity to express cytokines (28, 45). Therefore, to determine the Th type cytokine production of the Lin− CD123+ CD127low population, the expression of intracellular cytokines in activated PBMCs was assessed. The Lin− CD123+ CD127low population expresses IFN-γ, IL-2, and IL-4 after PMA/ionomycin activation (Figure 5A). Of note, the population that mainly expresses the cytokines downregulates CD123. The cytokine expression pattern was similar in the classic CD127+ ILC population (Figure 5B). Remarkably, the expression of IL-17 or IL-22 was not identified in the PB Lin− CD123+ CD127low population, and only a minor percentage of IL-22+ in the CD127+ ILC was identified. Considering that it has been reported that ILC may be activated by cytokines, PBMCs were cultured with cytokine cocktails for ILC1, ILC2, or ILC3 activation. Figures 5C,D indicate that IFN-γ is expressed in the Lin− CD123+ CD127low and CD127+ populations after culture with IL-12 and IL-15. The percentage of IFN-γ+ cells is similar in both populations; however, the expression of IFN-γ is increased in the CD127+ compared with the Lin− CD123+ CD127low ILC.
population. The culture with the ILC2 cocktail (IL-33 and IL-2) did not induce the expression of ILC2 type cytokines, such as IL-4 or IL-13, and the culture with the ILC3 cocktail (IL-1β, IL-2, and IL-23) did not induce the expression of ILC3 type cytokines, such as IL-17 or IL-22 (Figure S3 in Supplementary Material). These findings indicate that the steady-state peripheral Lin− CD123+ CD127low population expresses limited Th type cytokine variety.

The Lin− CD123+ CD127low Population in PB Expresses High Levels of IL-8
We subsequently isolated the Lin− CD123+ CD127low population from the lymphoid region and CD127+ ILC population to evaluate the cytokines in the supernatants via a multiplex assay after activation. Surprisingly, the Lin− CD123+ CD127low population produced high levels of IL-8 and low levels of IL-4 (Figure 6A), whereas the CD127+ expressed IL-8 and IL-2; however, other Th type cytokines were not identified in the purified populations (data not shown). The high expression of IL-8 by the CD123+ CD127low population was confirmed via intracellular detection in which IL-3 induced the expression of IL-8 (Figure 6C), which was significantly increased in the presence of PMA/Ionomycin (Figures 6B,C). The percentage of IL-8+ cells was significantly increased in the Lin− CD123+ CD127low cells compared with the CD127+ ILC (Figures 6B,C). The lack of expression of IL-2 and IFN-γ in the isolated Lin− CD123+ CD127low population was also confirmed via intracellular detection, in which only the expressions of IL-4 and IL-8 (Figure 6D, lower panel) and not IL-2 or IFN-γ were identified (Figure 6D, upper panel). These findings indicate that the freshly isolated steady-state Lin− CD123+ CD127low population expresses high levels of IL-8 and confirm the limited expression of Th type cytokines.
The Lin⁻ CD123⁺ CD127low CLA⁺ Population Has Migratory Potential Mediated by SDF-1

Considering the high display of CLA in the Lin⁻ CD123⁺ CD127low population, the expression of other homing receptors was subsequently assessed. Figure 7A indicates that the Lin⁻ CD123⁺ CD127low population exhibited an increased expression of CXCR4 and CD62L compared with the CD127⁺ ILC. In contrast, we identified an increased expression of CCR6 in the CD127⁺ ILC compared with the Lin⁻ CD123⁺ CD127low cells (Figure 7A). The Lin⁻ CD123⁺ CD127low population from PB highly expresses CXCR4; thus, migration assays were performed with SDF-1, the ligand for CXCR4. As indicated in Figure 7B, the Lin⁻ CD123⁺ CD127low population from PB migrates in response to SDF-1. In addition, SDF-1 is present in the skin culture supernatants from CS, and a significant increase in SDF-1 was identified in the inflamed skin cultures obtained from the biopsies of patients with psoriasis (Figure 7C). Remarkably, a significantly increased migration in response to supernatants obtained from cell cultures from PS lesion biopsies was dependent on CXCR4-SDF-1 compared with supernatants from the CS (Figure 7D). In addition, the Lin⁻ CD123⁺ CD127low but not the CD127⁺ population transmigrated through activated endothelial, and this migration significantly increased in response to SDF-1 (Figure 7E). Interestingly, the Lin⁻ CD123⁺ CD127low population increased the expression of CD127 after contact with activated endothelial cells (Figure S5 in Supplementary Material). These findings indicate the high migratory capability of the Lin⁻ CD123⁺ CD127low CLA⁺ population and suggest that under inflammatory conditions, SDF-1 could promote skin infiltration of Lin⁻ CD123⁺ CD127low cells.
**The Lin^- CD123^+ CD127^low Population within the Lymphoid Region Downregulates CD123 and Basophil Markers upon Activation**

The majority of Lin^- CD123^+ CD127^low cells within the lymphoid region downregulates CD123 and basophil markers such as FceR, CCR3, and CD203c upon activation. In contrast, most CD123^+ FSC^int SSC^int counterparts maintain the expression of these molecules (Figures 8A–C).

Considering that a reduction of the antigen recognized by 2D7 has been reported in activated basophils, the expression of IL-8 and 2D7 was evaluated after activation with PMA/Ionomycin and by IgE crosslinking. Remarkably, the population expressing IL-8 is the CD123^low and not the one maintaining high expression of CD123. We did not observe IL-8 expression after IgE crosslinking. In contrast, both cell populations reduced the expression of 2D7 in response to PMA/Ionomycin and IgE (Figure 8D). These results clearly show important functional differences between the lymphoid Lin^- CD123^+ CD127^low population and the CD123^+ FSC^int SSC^int one, suggesting that the Lin^- CD123^+ CD127^low population from the lymphoid region may require activation to acquire ILC functional features and to diminish basophil activity.

**Barrier Tissues, Such As Skin, Are Normally Infiltrated by a Specialized Lin^- CD123^low CD127^int Population with ILC Features**

Considering the high expression of CLA in the Lin^- CD123^+ CD127^low population and the downregulation of CD123 after activation next, we investigated whether an equivalent CD123^+ population might be identified in the skin. We found Lin^- CD123^low and Lin^- CD127^+ cells in the dermis of CS (Figure 9A). Notably, in the skin, the Lin^- CD123^low population exhibited an increased expression of CD127 and CD90 compared with the PB CD123^+ CD127^low population; however, it expressed lower levels of CD132 and CD161. The Lin^- CD123^low population in skin is positive for c-Kit, CRTH2, AhR, and IL-23 and expresses high...
levels of NKp44 compared with the CD127+ population. Although similar levels of CLA and CXCR4 are recorded (Figure 9B), the Lin− CD123low population does not express basophil markers such as FceR, CCR3, CD203c, or 2D7 (Figure 9C). Surprisingly, among the Lin− CD123+ population in the CS, ILC1, ILC2, and ILC3 (NKp44+ and NKp44−) may be identified by phenotype (Figure 9D). Among the CD127+ ILC population, ILC1, ILC2, and ILC3 (NKp44+ and NKp44−) were also identified (Figure 9E). However, the proportions are different compared with the Lin− CD123+ skin population and the PB CD127+ ILC, as well as between different CS samples (data not shown). Considering that the Th cytokine production by ILC has mainly been reported in peripheral tissues, the cytokine production by the CS Lin− CD123low population was subsequently evaluated. After activation, the Lin− CD123low population present in CS expresses IFN-γ, IL-2, and IL-4 and, remarkably, IL-17 and IL-22 (Figure 10A). The CD127+ ILC population exhibited a similar pattern of cytokine expression; however, the percentages were different between both populations (Figure 10B) and between different CS biopsies. As a result of the expression of IL-17 and IL-22 after PMA/Ionomycin activation, we subsequently assessed whether the Lin− CD123low population responded to the ILC3 cocktail (IL-1β, IL-2, and IL-23) in CS. CS was stimulated with the ILC3 cocktail, PMA/Ionomycin or the combination. Figure 10C indicates that the Lin− CD123low population exhibits a minor expression of IL-22 or IL-17 in response to the ILC3 cocktail; however, increases in the percentages of IL-22+, IL-17+, and both were identified in response to the combination of ILC3 cocktail and PMA/Ionomycin compared with only PMA/Ionomycin. The expression of IL-17 by the skin Lin− CD123low cells and the CD127+ populations correlates with the expression of RORyt (Figure S6 in Supplementary Material) and with the ILC3 subset phenotype. Regarding the IL-8 expression, we determined that after stimulation, the Lin− CD123low population from CS expressed IL-8 to a lesser extent compared with the PB Lin− CD123+ CD127low population (Figure 10A). These findings indicate that in accordance with the high expression of CLA in the Lin− CD123+ CD127low population from PB and with the downregulation of CD123 after activation, we identified a Lin− CD123low population in CS that exhibit increased phenotypic and Th type cytokine diversity compared with its PB counterpart. These findings strongly suggest that specialized Lin− CD123low CD127+ population with ILC features normally infiltrate barrier tissues, such as skin, which appear to be in a late stage of activation.

**SDF-1 Dependent Migration of Lin− CD123+ CD127low May Precede Activation and Local Production of IL-17 and IL-22 in Psoriasis Patients**

As a result of the migratory potential and the ability of the Lin− CD123low CD127int population present in CS to express IL-17 and IL-22, the frequencies and the expression of these cytokines by the ILC-related populations from psoriasis patients were subsequently assessed. Importantly, a significant increase in the frequencies of the Lin− CD123+ CD127int population was identified in the skin lesions of psoriasis patients compared with the CS. Interestingly, an increase was also identified in the non-lesioned (NL) skin of psoriasis patients. We also identified an increase in the CD127+ population in the NL and lesioned skin from psoriasis patients (Figure 11A). The Lin− CD123low population in the CS expressed IL-17 and IL-22 only after activation. However, in some patients, the Lin− CD123low CD127int population from the lesioned and NL skin expressed IL-17 and IL-22, even in the absence of additional stimulation (Figure 11B). The expression of IL-17 was significantly increased in the skin lesions of the psoriasis patients both in the absence of additional stimulation and after stimulation compared with the unstimulated CS (Figure 11C).
The expression of IL-22 in the psoriasis patients was significantly increased after stimulation compared with the non-stimulated CS (Figure 11D). The percentage of cells that expressed IL-22 and notably IL-17 was increased in the Lin− CD123low population compared with the CD127+ population (Figures 11C,D). These findings strongly suggest that SDF-1-dependent migration...
of Lin− CD123+ CD127low cells may precede the activation the downregulation of Lin− CD123 and local production of IL-22 and remarkably IL-17 in psoriasis patients.

**DISCUSSION**

In the past years, a high diversity of ILC, including non-classical populations, has been described. Here, we report a Lin− CD123+ CD127low population in PB that possesses ILC features. Moreover, IL-3 appears to be crucial for its maintenance and identity. The Lin− CD123+ CD127low population highly expresses CLA and has skin-homing potential. Moreover, a similar population CD123+ was identified in the skin, which likely participates in the pathogenesis hallmarks of psoriasis.

Recently, different transcription factors have been described as crucial for ILC identity and development. NFIL3, which is regulated by IL-7, is crucial for ILC development (16, 19). Classic ILC express IL-7Rα (CD127), although CD123 (IL-3Rα) is usually used to exclude basophils during ILC identification; this report identified a Lin− CD123+ CD127low population that expresses basophil and lymphoid markers but remarkably expresses several ILC features, which appears to be regulated by IL-3.

Classic ILC express CD127; however, recently, it has been reported the presence of non-classical CD127− ILC populations and also an early ILC precursor (EILP) CD127low (8, 9). The Lin− CD123+ population we report here shows lymphoid morphology and expresses low levels of CD127. However, to explore the lymphoid origin of such population, we evaluated unproductive DJ rearrangements as a molecular fingerprint of early lymphoid progenitors. There were no apparent rearrangements neither in the CD127− classical ILC nor in the Lin− CD123+ CD127low population when compared with B-lymphocytes or in the acute B cell leukemia cell line NAL-M6. Nevertheless, the human ILC progenitor has only been described in tissues (46) and not in bone marrow or PB. Therefore, until now, the molecular features of the human ILC precursor are unknown. As an alternative to determine the lymphoid origin, the expression of CD7 was evaluated. Importantly, the Lin− CD123+ CD127low population expressed CD7, which has been reported to be expressed in the CLP and maintained in different lymphoid populations (3). Co-expression of CD127 and CD7 in the Lin− CD123+ population suggests lymphoid-related features. However, future studies are necessary to formally prove the lymphoid origin of this population.

CD123 is normally used to exclude pDCs, basophils, and mast cells when identifying ILC. However, the Lin− CD123+ CD127low population showed clear differences in the expression of MHC-II, BDCA-4, and BDCA-2 compared to pDCs. With regard to mast cell similarities, the low abundance of mast cells in PB under normal conditions suggest that the CD123+ CD127low population are not mast cells or their precursors, as they did not expressed CD34 (35). Nevertheless, in this report, we identified a mixture of lymphocyte-sized cells with no segmented nuclei (no lobes) and cells with classical basophil morphology (lobed nuclei). In addition, two populations of Lin− CD123+ cells were observed, the Lin− CD123+ CD127low population from the lymphoid region (FSClow/SSClow) and an FSCint SSCint CD123+ population. The Lin− CD123+ CD127low CD127low population from the lymphoid region expresses similar levels of the basophil markers FcεR and CCR3, compared to the CD123+ FSCint SSCint region. However, it expresses lower levels of CD203c and of the antigen recognized by mAb 2D7, which is expressed in basophil granules, indicating the expression of granules in this population (43). Our results show then, a distinct Lin− CD123+ CD127low population within the lymphoid region with ILC properties that transiently share some features with CD123basophils from the FSCint SSCint region. Importantly, such population decreases the expression of basophil markers upon activation, whereas most CD123+ cells from the FSCint SSCint region maintains the expression of CD123 and show more stable basophil marker display after activation. Of special interest for our future investigations, subfractioning the two subsets described in this study will be highly relevant
for further transcriptional analyses at the clonal level. Whether basophils and the Lin− CD123+ CD127low population develop from a common progenitor, or their shared phenotypic properties only resemble the phenomenon referred to as lineage priming where "promiscuous expression of several lineage-affiliated genes precedes lineage commitment but does not alter the biological potential" as described for some oligo- or bipotential precursors (47), is still a matter in question.

Remarkably, in this report, several evidences support the finding that the Lin− CD123+ CD127low from the lymphoid region population possesses several ILC features. First, the expression of low levels of CD127 by protein and mRNA. Second, the observed expression of CD7, which is related to CLP and maintained in different lymphoid cells, including the classical ILC. Third, the high expression of CD132, which has been reported as crucial for ILC development (48). Fourth, the expression of CD90, a classical ILC marker as well as other ILC markers such as CD161, α4 integrin which has been described in ILC precursors, and the expression of c-Kit, CRTH2, AhR, IL-23R, and CCR6. Fifth, the expression of several transcription factors such as Id2, NFIL3, TOX, PLZF described for ILC identification in humans (16, 17, 34) and TCF-1, recently described for ILC development in mice (15, 49), and sixth, the increase in NFIL3, TOX, and PZLF expression by IL-3 suggesting that NFIL3 might regulate the identity of the ILC-related features in the Lin− CD123+ CD127low population. Thus, our data indicate that the use of anti-FcεR and anti-CD123 in the lineage cocktail in previous reports may have limited the identification of an alternative population with ILC features in PB.

In human PB, classical ILC express CD161 and exhibit phenotypic diversity (ILC1, ILC2, and ILC3 NKp44−). Interestingly, the PB Lin− CD123+ CD127low population comprises a majority conspicuous population (CD161low, c-Kit+, and CRTH2+), which, by phenotype, is similar to ILC2 and a minority of ILC1 and ILC3 populations. This finding was inconsistent with the low expression of GATA-3 and the expression of T-bet and RORγt. GATA-3 has been reported as crucial for classical ILC development (50, 51). Moreover, it has recently been reported that a common ILC progenitor RORγt+ present in secondary lymphoid tissue has the potential in vitro to give rise to all human ILC subpopulations (46). These findings support the idea that the expression of RORγt in the Lin− CD123+ CD127low population in PB may be related to a further process of differentiation and diversification of this population in peripheral tissues. In contrast, in the classical CD127+ ILC population in PB, similar to other reports (39–41), ILC1, 2, and 3, as well as the expression of T-bet, RORγt, and GATA-3,
were identified. These findings indicate that both populations share several ILC features; however, the Lin− CD123+ CD127low population in PB may be in a different stage of differentiation and it may require different transcriptional factors and cytokines for development.

It has been reported that ILC express cytokines similar to the Th lymphocytes in peripheral tissues (4, 52). However, there are few reports in PB and most of the studies use cell lines derived from ILC obtained from patients (7, 39, 40). The freshly isolated Lin− CD123+ CD127low population expresses only IL-4 after activation and the classical CD127+ population expresses IL-2 (data not shown). However, among the total PBMCs, the Lin− CD123+ CD127low and the classic CD127+ ILC populations expressed IFN-γ in response to PMA/Ionomycin and the ILC1 activation cocktail. However, even in the presence of IL-1β, IL-2, and IL-23, the Lin− CD123+ CD127low and CD127+ ILC did not express IL-17 or IL-22, which indicates that steady-state PB ILC populations may require further differentiation or activation to express all Th type varieties of cytokines. Importantly, the Lin− CD123+ CD127low population highly expresses IL-8 compared with the classical CD127+ ILC, notwithstanding that IL-8 expression by ILC has only been assessed in a limited number of reports (6, 53), and the function of this cytokine expression in PB ILC has not been investigated. Interestingly, the population that expresses cytokines after activation shows a downregulation of CD123, therefore further examination of the phenotype and function of the Lin− CD123+ CD127low population after activation was evaluated. Interestingly, the population downregulating CD123 expresses IL-8 and decreases the expression of basophil markers, including 2D7. Of note, these effects were not observed in the population that maintains the CD123 expression or after IgE crosslinking. However, upon IgE activation, an important decrease in the 2D7 expression was observed in the Lin− CD123+ CD127low population, suggesting the activity of this population in response to IgE. These results suggest that activation of the Lin− CD123+ CD127low cells may precede their capability of acquiring ILC function and diminishes basophil activity.

In mouse models, it has been proposed that the complete differentiation of ILC occurs in peripheral tissues (15,49). In human tonsils, the identification of an ILC3 subpopulation that presents a “naïve” phenotype has been recently described; these cells were unresponsive to IL-23 and IL-1β, despite their expression of IL23R and IL1R1 transcripts, and were characterized.

**FIGURE 10** Skin Lin− CD123low cells produce IL-17 and IL-22. (A,B) Percentages of positive and negative total skin cells that express IFN-γ, IL-2, IL-4; IL-17, IL-22, and IL-8 in the presence of PMA/Iono (6 h) in the Lin− CD123low (A) and Lin− CD127+ (B) populations within total control skin cells. Upper panels: FMO controls. (C) Expression of IL-17 and IL-22 in the Lin− CD123low skin cells cultured for 18 h in the presence of IL-1β, IL-2, and IL-23 (ILC3 activation cocktail) + PMA/Iono (last 6 h) or ILC3 activation cocktail + PMA/Iono (last 6 h). Density plots are representative of at least three independent experiments. FMO, fluorescence minus one control.
by the expression of CD62L and CD45RA (23). Our findings demonstrated that the Lin^− CD123^+ CD127^low^ population in PB has limited Th cytokine production and highly expresses homing molecules (CLA, CXCR4, and CD62L) and also a IL23R and AhR, which, in addition to the expression of RORyt, suggests that this population may be in an early stage of differentiation with the potential to migrate into different tissues to be fully differentiated.

Importantly, an equivalent of the CD123^+^ population was present in the CS that expresses several ILC features (CD127^{int}, CD132, CD90, c-Kit, CRTH2, AhR, IL-23R, NKp44, and CCR6), where an increased phenotypic diversity (ILC1, ILC2, and ILC3) was also identified. Consistent with this diversity, more Th type cytokines, including IL-22 and remarkably IL-17, were identified in the skin Lin^−^ CD123^+^ population after activation.
The expression of IL-22 and IL-17 was consistent with the high expression of IL-23 and AhR (54) in the Lin−CD123low population. This finding suggests that in peripheral tissues, this population may express a wider variety of cytokines as reported for other ILC subsets (24, 28). These findings also support that in the skin, the CD123+ population becomes fully differentiated, as proposed in the mouse model for the classical ILC subsets (15, 55). The cytokine expression by the direct isolated skin ILC was not evaluated; therefore, it is possible that other activated cells in the skin cultures may contribute to the cytokine expression by the Lin−CD123low CD127low population, like has been described for ILC2 in skin (56). Remarkably, the Lin−CD123low population in skin expresses IL-8, which may be relevant in the recruitment of other innate cells, such as neutrophils (57). Similar to the PB, the CD123+ population in the skin is more frequent than the classical CD127+ ILC. Therefore, it is possible that the high expression of CLA may be involved in non-inflammatory skin homing as reported for T cells (58) and ILC, in which CLA expression in ILC and cytokine production in psoriasis patients even in the NL skin of psoriasis patients (40). Our findings indicate that the CXCR4/SDF-1+CD123+ CLA+ population identified in PB may represent a steady-state reservoir with the potential to migrate into the skin, mediated by CLA, and suggest that barrier tissues, such as skin, are normally infiltrated by specialized Lin−CD123low CD127low CD123+ populations with ILC-related features.

In addition, we demonstrated that the Lin−CD123+ CD127low population transmigrates in response to SDF-1 and in the presence of activated endothelial cells mediated by SDF-1. Similar to other reports (59, 60) and other inflammatory diseases (61), an increase in the levels of IL-1 in the supernatants of psoriasis patients was identified; this finding suggests that the Lin−CD123+ CD127low CLA+ population identified in PB may represent a steady-state reservoir with the potential to migrate into the skin, mediated by CLA, and suggest that barrier tissues, such as skin, are normally infiltrated by specialized Lin−CD123low CD127low CD123+ populations with ILC-related features.

Importantly, both the Lin−CD123low and CD127+ populations were increased in the skin of psoriasis patients; however, it is possible that the CD127+ ILC may migrate by a mechanism independent of CXCR4/SDF-1 but CCR6-dependent because high levels of CCR6 were identified in the CD127+ ILC population. Remarkably, both populations express IL-22 and IL-17 in the skin of psoriasis patients and represent two hallmark cytokines in the immunopathology of psoriasis in mouse models and humans (64–66). The expression of these cytokines was increased in the Lin−CD123low population compared with the CD127+ ILC. It has been reported that a CD3+ population expresses IL-17 in the PB and skin of psoriasis patients (41). However, there is only one report of a slight production of IL-17 by classic NCR+ ILC3 from the skin of psoriasis patients (40). Our findings indicate that the Lin−CD123low population may be an important and additional innate source of IL-22 and, importantly, IL-17 in the lesional and probably in the NL skin of psoriasis patients. In addition, our findings suggest that the SDF-1-dependent migration of Lin−CD123+ CD127low cells from the PB to the skin may precede the activation and local production of IL-17 and IL-22 in psoriasis patients.

In summary, according to the proposed model (Figure 12), we have identified in PB a novel Lin−CD123+ CD127low population with a mixture of lymphoid (CD127low CD7low) and basophil (FceR, CR3, CD203d 2D7low) properties that possesses several ILC features, including the phenotype Id2+ NFIL3+ PLZF+ TOX+ TCF-1+ CD132+ CD90+ CD161+α4 integrin+ c-Kit+ CRTH2+AhR+IL-23R+ CCR6+, and high migratory capabilities. IL-3 appears to comprise a crucial growth factor for survival of
the ILC-related features in the Lin−CD123+ CD127low population. A similar but specialized Lin− CD123mm population normally infiltrates barrier tissues, such as skin. We propose that CXCR4/ SDF-1 is an important skin-homing mechanism under inflammatory conditions in psoriasis. The increase of the Lin− CD123mm population in the NL and lesional skin of psoriasis patients supports its high migratory potential. Remarkably, expression of IL-22 and particularly IL-17 by the Lin− CD123mm population in the skin of psoriasis patients strongly suggests that this population may contribute to the immunopathological hallmarks of a skin disease such as psoriasis.

ETHICS STATEMENT

The study was approved by the local ethic committee from the Centro Dermatologico Ladislao de la Pascua Registry number: 112/2016 and by the ethic committee for Health Research from Instituto Mexicano del Seguro Social (IMSS) (committee number:3601). The study was conducted according to the principles detailed in the Declaration of Helsinki. All participants signed an informed consent form.

AUTHOR CONTRIBUTIONS

LB conceived and directed the project. LB, LM-V, and OC-E designed the experiments. LM-V, OC-E, AM, CA-F, and MV-A performed the experiments. LM-V and OC-E acquired and analyzed the data. LB and RP contributed reagents/materials/analysis tools. LB, LM-V, and RP wrote the manuscript. CM-G and FJ-S were involved in the recruitment and diagnosis of psoriasis patients. EF-O provided the control skin biopsies. M-TL realized the Quantitative RT-PCR and JM-B and JT-S realized the incomplete DH-JH rearrangements analysis. All the authors reviewed critically the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00176/full#supplementary-material.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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