Idiopathic CD4+ T-cell lymphocytopenia is associated with impaired membrane expression of the chemokine receptor CXCR4

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Abbreviations: BM: Bone marrow; CHX: Cycloheximide; GPCR: G protein-coupled receptor; HPC: Hematopoietic progenitor cell; ICL: Idiopathic CD4+ T-cell lymphocytopenia; IL-2: Interleukin-2; MANO: mannoprotein antigen from Cryptococcus neoformans; MFI: Mean Fluorescence Intensity; PPD: Purified protein derivative; SEB: Staphylococcus aureus Enterotoxin B; SDF-1: Stromal cell Derived Factor-1; TT: Tetanus anatoxin; WS: WHIM syndrome.
ABSTRACT

Idiopathic CD4+ T-cell lymphocytopenia (ICL) is a rare acquired T-cell immunodeficiency of unknown pathogenic basis. Six adults with ICL who developed opportunistic infections were investigated using extensive immunophenotyping analysis and functional evaluation of the chemokine receptor CXCR4. For all 6 patients studied, a profound defect in CXCR4 expression was detected at the surface of CD4+ T lymphocytes, in association with an abnormal intracellular accumulation of CXCR4 and of its natural ligand, the chemokine CXCL12. For all patients studied, CD4+ T-cell chemotactic response towards CXCL12 was decreased, while sensitivity to CXCL8 was preserved. CXCR4 recovery following ligand-induced endocytosis was impaired in ICL CD4+ T-cells. Upon in vitro addition of IL-2, membrane expression of CXCR4 returned to normal levels in 5/6 patients, while intracellular accumulation of CXCR4 and CXCL12 disappeared. Upon therapeutic administration of IL-2, CD4+ T-cell count and membrane CXCR4 expression and function improved over time in three of four patients treated. Therefore, our data indicate that ICL is associated with defective surface expression of CXCR4, which may be reversed by IL-2.
INTRODUCTION

Idiopathic CD4+ T-cell lymphocytopenia (ICL) is characterized by a profound and persistent CD4+ T-cell defect which predisposes to opportunistic infections1-5. ICL definition includes an absolute CD4+ T-cell count <300 cells/mm^3 or <20% of CD4+ T-cells on more than one occasion at least 6 weeks apart, together with lack of other known immune defects6. There is no clear evidence for an infectious or environmental cause of the disease3,7. Heterogeneous immunologic profiles have been reported so far in ICL patients1,8,9. Interleukin-2 (IL-2) has been reported to increase CD4+ T-lymphocyte count and improve the outcome in a few ICL patients10,11.

Mechanistic studies of T-cell function in ICL remain scarce. Decreased T-cell responses as well as increased T-cell activation have been reported7,12. CD8+ T-cell counts remain in the normal range or are often decreased in ICL13. Functional investigations have revealed an increased propensity of ICL T-cells to undergo apoptosis, a process partially dependent on Fas expression14,15. Markers for activation and turnover are increased in CD4+ T-cells but not in CD8+ T-cells, pointing at a specific alteration of the CD4+ T-cell compartment13. Another factor that may contribute to the CD4+ T-cell defect is a decreased clonogenic capacity of the bone marrow (BM) in ICL patients16. A frequent alteration observed in ICL consists in increased levels of IL-7 in peripheral blood, consistent with the triggering of a homeostatic response to restore normal CD4+ T-cell counts9,17.

Chemokines are secreted proteins that govern the migration of leukocyte subsets to their specific niches within lymphoid organs and inflammatory sites18,19. Chemokines mediate their functions by binding to chemokine receptors, which belong to the heptahelical G protein-coupled receptor (GPCR) family20. The chemokine Stromal cell-Derived Factor-1 (SDF-1/CXCL12), the sole natural ligand for the broadly expressed GPCR CXCR4, acts as a chemoattractant for hematopoietic progenitor cells (HPCs) and leukocytes (reviewed in21).
CXCL12 is constitutively produced by stromal, epithelial and endothelial cells, notably in lymphoid organs. In postnatal life, CXCL12/CXCR4 interactions regulate the BM homing and retention of HPCs, the trans-endothelial migration of leukocytes, as well as their lymphoid and peripheral trafficking\textsuperscript{22-25}. In the thymus, CXCL12 expression by epithelial cells is required for the settling, survival/expansion and subsequent differentiation of CXCR4\textsuperscript{+} early T-lymphoid progenitors (reviewed in\textsuperscript{26}). In the periphery, the CXCL12/CXCR4 axis contributes to the homing, positioning and activation of T-cells within secondary lymphoid tissues (reviewed in\textsuperscript{27,28}). Due to these pleiotropic effects, alterations of CXCR4 expression or activity are likely to severely impact T-cell differentiation and trafficking. Supporting this notion, mice reconstituted with progenitor cells expressing a CXCL12 intrakine, which binds intracellularly to CXCR4 and prevents its membrane expression, display a T-cell lymphopenia together with an impaired intrathymic maturation\textsuperscript{39}. Conversely, transgenic mice overexpressing a human CXCR4 gene in CD4\textsuperscript{+} T-cells harbor a severe CD4\textsuperscript{+} T-cell depletion in peripheral blood, with a concomitant increased homing of these cells in the BM\textsuperscript{30}. These studies clearly indicate that modulation of CXCR4 expression or function disturbs CD4\textsuperscript{+} T-cell trafficking within tissues.

The molecular basis of ICL is still unknown. We hypothesized that expression or function of CXCR4 could be altered in ICL CD4\textsuperscript{+} T-cells. We provide evidence for defective CXCR4 expression and function in circulating T lymphocytes from six patients with ICL, and analyze the restoration of these defects by IL-2 therapy.
PATIENTS AND METHODS

Subjects

Six adult patients were referred to us for investigation of a first opportunistic infection occurring in the setting of CD4+ T-cell counts persistently <300/mm³ (Table 1). All patients were seronegative for HIV-1 and -2, HTLV-1 and -2, HBV, HCV and HHV-8. HIV load was undetectable and patients had no clinical and biological evidences of active EBV or CMV infections, SLE or sarcoidosis. All patients met the definition of ICL6 and none mentioned a familial history of severe infections. We obtained authorization from the French governmental agency (Agence Française de Sécurité Sanitaire des Produits de Santé), which delivers authorization for drug use beyond approved usages, to treat 4 of the 6 patients (P1, P2, P3 and P6) with recombinant IL-2 (one course consisting in 4.5 millions units b.i.d. subcutaneously for five days, every 6 weeks) for at least 8 cycles31. Tolerance of IL-2 was considered acceptable enough to repeat therapeutic cycles, although all patients experienced some side effects32. The patients’ informed consent was obtained for molecular and immunological investigations and IL-2 therapy in accordance with the Declaration of Helsinki. The control group included 12 healthy volunteers aged 18-60 years of both genders, who were non-smokers, seronegative for HIV, HBV or HCV, without evidence of cancer, congenital heart disease or connective tissue disorder, and who had all received BCG vaccination.

Sample processing and cell culture

Blood samples were drawn several months after effective cure to investigate the immune defect and the molecular basis of ICL. All experiments were performed within 3 hours following blood sampling. Samples from healthy controls were processed in parallel. PBMC were isolated from heparin-treated blood samples using Ficoll-Paque™ Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Whole blood and PBMC were
used either right after collection or after overnight incubation at 37°C with 9% CO₂ in RPMI 1640 culture medium supplemented with 10% heat-inactivated human AB serum (Valbiotech, France) or FCS in the presence or absence of recombinant IL-2 (10 UI/ml, Peprotech Inc., NJ, USA). Because of a complex experimental design (patients living in remote areas requesting to combine outpatient visit, sampling and experiments), the number and identity of the patients varied for some of the analyses (specified in the corresponding sections).

**Immunophenotypic analyses**

Absolute counts were determined by four-color flow cytometry analyses (EPICS-XL MCL flow cytometer, Coulter Electronics Inc., Hialeah, FL) on whole blood samples with fluorescent beads used as an internal standard. Seven Ab combinations were used for analysis of leukocyte subsets: 1. CD45-FITC, CD3-PECy5, CD4-RD1, CD8-ECD; 2. CD4-ECD, CD8-PECy5, CD38-RD1, HLA-DR-FITC; 3. CD4-ECD, CD8-PECy5, CD25-RD1, CD28-FITC; 4. CD4-ECD, CD8-PECy5, CD62L-RD1, CD45RA-FITC; 5. CD19-ECD, CD8-PECy5, CD56-RD1, CD16-FITC; 6. CD4-ECD, CD8-PECy5, CXCR4-PE, CCR5-FITC; 7. CD3-ECD, CD8-PECy5, TCRαβ-RD1, TCRγδ-FITC. All monoclonal Abs (mAbs) were from Beckman Coulter except those specific for CXCR4 (clone 12G5) or CCR5 (clone 2D7) (BD Biosciences, San Diego, CA). Naive T-cells were defined by the co-expression of CD45RA and CD62L markers. Cells not co-expressing CD45RA and CD62L markers were considered as memory T-cells and those expressing CD38 and/or HLA-DR as activated T-cells.

Intracellular staining was performed in PBMC to detect CXCR4, CCR5 or CXCL12. After incubation for 30 min with saturating amounts of non-conjugated Abs against CXCR4 and CCR5, cells were washed twice in PBS, fixed with 2% formaldehyde solution, and then treated for 10 min at 25°C with a permeabilization buffer (0.5% saponin, 5% FCS in PBS).
CCR5 (FITC) and CXCR4 (PE) conjugated mAbs or the K15C anti-CXCL12 mAb, followed by an anti-mouse FITC conjugated donkey polyclonal Ab, were then added for 30 min at 25°C. Cells were analyzed after further washes. Leukocytes from 12 healthy donors were used for the control group.

**In vitro lymphocyte proliferation assays**

PBMC grown in 96-well culture plates at 1x10^5 cells per well were stimulated with 0.5 µg/ml of *Staphylococcus aureus* Enterotoxin B (SEB, Sigma), as a positive control, tetanus anatoxin (TT) (10 µg/ml), candidin (20 µg/ml), purified protein derivative from *Mycobacterium tuberculosis* (PPD) (5 µg/ml, Statens Institut, Copenhagen, Denmark) and the mannoprotein antigen from *Cryptococcus neoformans* (MANO) (20 µg/ml) (a gift from S.M. Levitz, University of Massachusetts Medical School, MA). Proliferation was quantified by flow cytometry, after labeling cells with 10 µM 5(6)-CFSE (Molecular Probes) before culture. Fluorescence intensity loss due to division cycles was determined after 6 days of culture (% CFSE<sub>low</sub> cells). Background proliferation was <3%. Control values were obtained from 25 healthy volunteers, except for MANO stimulation (5 subjects).

**Cytokine secretion assay**

IFN-γ and TNF-α secretion were measured in PBMC-derived supernatants after 36 hours of culture in the presence of SEB, TT, candidin, PPD or MANO. Cytokines were detected by flow cytometry using the CBA technique (BD Biosciences). All analyses were performed with the Statistica 8.0 software, using the Mann-Whitney U nonparametric statistical test. Significant differences between groups (p<0.05) were reported on data plots.
**Chemotaxis assays**

Chemotaxis was performed using 24-well chemotaxis chambers with polycarbonate filters (3 µm pore size) as described\textsuperscript{35,36}. Briefly, diluted recombinant CXCL12 (chemically synthesized and provided by F. Baleux, Unité de Chimie Organique, Institut Pasteur, Paris, France) at various concentrations or recombinant CXCL8 (R&D Systems, France) at 30 nM were added to the lower chamber. PBMC that migrated to the lower chamber after 2 h incubation were collected, stained with CD45, CD4, CD8 and CD3 mAbs and counted by flow cytometry. Cells migrating across the membrane were calculated as follows: \[
\frac{\text{number of cells migrating to the lower chamber in response to the chemokine} - \text{number of cells migrating spontaneously}}{\text{number of cells added to the upper chamber at the start of the assay}} \times 100.
\]

**CXCR4 internalization and recycling assays**

CXCR4 internalization was studied as described\textsuperscript{36,37}. Briefly, PBMC (2x10\textsuperscript{6} cells/ml) were incubated for 40 min at 37°C with 200 nM CXCL12 in the presence of 50 µg/ml cycloheximide (CHX, Sigma). For CXCR4 recycling assays, PBMC were washed in acidic glycine buffer (pH=2.7) and further cultured for up to 120 min at 37°C with CHX in the absence of CXCL12. Levels of membrane CXCR4 expression were determined by flow cytometry (FACSCalibur\textsuperscript{®}, BD Biosciences) using the PE-conjugated anti-human CXCR4 (clone 12G5) or the corresponding isotype control Ab in combination with FITC-conjugated anti-human CD3 (clone SK7) and APC-conjugated anti-human CD4 (clone RPA-T4) mAbs (BD Biosciences). CXCR4 expression in stimulated cells was calculated as follows: \[
\frac{\text{CXCR4 geometric mean fluorescence intensity [MFI] of treated cells}}{\text{CXCR4 geometric MFI of unstimulated cells}} \times 100.
\]
RESULTS

Immunological features of patients with ICL

All patients presented with CD4+ T-cell lymphocytopenia, an increase in NK- and γδT-cell populations, and a decrease in B-cell population which reached undetectable levels for 2 patients (P1 and P3) (Table 1). For all patients, serum levels of IgG, IgM and IgA were within the normal range (Table 1). Of note, CD4+ T-cell depletion has persisted for over 18 months after the diagnosis of lymph node tuberculosis for P5. Proportions of ICL naive CD4+ (35.3±27.3%) and CD8+ (49.8±22.8%) T-cells did not differ significantly from those of controls (41.4±19.5% and 46.3±8.4%, respectively). In ICL, 17.3±5.6% of total lymphocytes, 41.2±15.9% of CD4+ T-cells and 3.2±1.4% of CD8+ T-cells expressed the CD25 marker, while the activation markers CD38 and/or HLA-DR were expressed in all populations at normal levels (data not shown). Proliferation of CD4+ and CD8+ T-cells in the presence of TT, candidin and PPD was consistently lower in ICL patients than in controls, while proliferation to the superantigen SEB was normal (Figure 1, top panels). As expected, MANO proliferation was observed only in ICL T-cells. After specific stimulations, a decreased in the production of IFN-γ and TNF-α by ICL PBMC was observed (Figure 1, bottom panels). These findings provide evidence for severely compromised T-cell responses but normal immunoglobulin production in ICL patients.

Abnormal CXCR4 and CXCL12 detection in ICL CD4+ T-cells

Membrane CXCR4 expression was analyzed in whole blood samples. For all patients, CD4+ T-cell lymphocytopenia (Figure 2A) was associated with a profound decrease in CXCR4 expression at the surface of CD4+ T-cells (5.7±3.6% vs 25.0±5.0% in controls, p<0.0001) and CD8+ T-cells (6.4±3.8 vs 19.0±8.0% in controls, p<0.0001) (Table 1 and Figure 2B). Of note, CXCR4 down-regulation was more pronounced within the CD4+ than the CD8+ T-cell subset.
Membrane CXCR4 expression was found to be markedly decreased in both naive and memory CD4+ and CD8+ T-cell subsets (Supplemental Figure S1). In contrast, CXCR4 expression was preserved at the surface of monocytes (data not shown), suggesting a T-cell-specific defect. We found that lack of CXCR4 expression was not due to masking by a ligand, as cell washing in acidic glycine buffer (pH=2.7) did not change CXCR4 expression levels (data not shown). Expression levels of CCR5 in ICL CD4+ and CD8+ T-cells were in the normal range or even increased (Table 1, Figure 2B, and supplemental Figure S1), indicating a CXCR4-specific rather than a global chemokine receptor defect. After overnight incubation with recombinant IL-2, expression levels of CXCR4 at the surface of CD4+ T-cells from 5 out of 6 patients were similar to those detected in control cells (Figure 2B). For the last patient (P3), CXCR4 remained barely detectable at the CD4+ T-cell surface after in vitro IL-2 treatment.

Loss of membrane CXCR4 expression was accompanied by an abnormal intracellular accumulation of CXCR4 (41.8±11.3% vs 2.0±0.6%, p<0.0001) and of CXCL12 (39.0±15.9% vs 0%, p<0.0001) in CD4+ T-cells from all patients (Table 1 and Figure 2C). In addition, an abnormal intracellular detection of CXCR4 was found in both naive and memory CD4+ and CD8+ T-cell subsets (Table 1 and Supplemental Figure S1). IL-2 therapy was initiated in 4 patients. At the end of the first IL-2 course, surface expression levels of CXCR4 were increased in CD4+ T-cells from 3 patients, namely P1, P2 and P6 (Figure 2B). Membrane recovery of CXCR4 was associated with a disappearance of intracellular CXCL12 (Figure 2C) and CXCR4 (data not shown). For P3, membrane expression of CXCR4 remained low following in vivo IL-2 administration, as it had after in vitro IL-2 stimulation (data not shown).
**Lack of modulating effects of ICL plasmas on membrane CXCR4 expression**

To gain insight into the mechanism underlying the abnormal intracellular accumulation of CXCR4 in ICL T-cells, CXCL12 levels were measured in plasmas from healthy and ICL (P1 and P6) subjects by immunoassay. No differences were found between patients and controls (data not shown). As polymorphisms in *CXCR4* and *CXCL12* genes have been associated with pathological disorders (reviewed in\(^{38}\)), we looked for possible mutations associated with ICL. Sequencing of cDNA products amplified from PBMC (for *CXCR4*) or BM aspirates (for *CXCL12* isoforms) of two patients (P1 and P3) did not reveal mutations in the *CXCR4* open reading frame or in those of *CXCL12-α* and *-β* (data not shown). These findings do not support the notion that anomalies in CXCL12 structure or production account for the sustained CXCR4 internalization and the loss of CXCR4 expression at the surface of ICL CD4\(^+\) T-cells.

We next looked for the presence of cytokines and chemokines known to modulate CXCR4 expression in the plasma of ICL patients by multiplexed immunoassay. The panel included molecules known to down-regulate CXCR4 (*e.g.* IFN-\(α\), IFN-\(γ\)) or to up-regulate CXCR4 (*e.g.* inflammatory cytokines and chemokines, \(γ\)-c cytokines) in T lymphocytes\(^{39-42}\). The levels of 25 analytes measured in P6 were in general within the range of values found in 11 healthy donors (Supplemental Table 1). We also investigated whether the plasma of ICL patients could induce CXCR4 down-regulation in normal PBMC. Membrane CXCR4 expression was measured in healthy donor PBMC incubated overnight with 30% plasma from ICL or heterologous healthy subjects. We did not detect a major difference between the effects of plasmas from 2 ICL patients (P1 and P6) and 4 healthy donors (Supplemental Figure S2). Overall, we have not identified so far a component of ICL plasmas that could account for CXCR4 down-regulation.
Impaired CXCL12-promoted chemotaxis of ICL T lymphocytes

Impaired surface expression of CXCR4 may alter the responsiveness of T lymphocytes to CXCL12. This possibility was investigated in leukocytes from ICL (P1, P2 and P6) and healthy subjects (H1, H2 and H3) using a chemotaxis assay. Addition of CXCL12 resulted in a dose-dependent chemotactic response of control and ICL total lymphocytes (Figure 3A), CD4+ and CD8+ T-cells (Figure 3B and C). However, ICL T-cells displayed weaker migratory responses at all concentrations, indicating a lower efficiency of chemotaxis to CXCL12. These findings are consistent with the decreased expression levels of CXCR4 observed in ICL T-cells. No alteration of CXCL12-promoted chemotaxis was observed in ICL leukocytes other than T-cells (data not shown). Importantly, no impairment in chemotactic response to CXCL8 was detected in ICL leukocytes, independent of the subset considered, including CD4+ or CD8+ T-cells (Figure 3B and C), non-T lymphocytes and monocytes (data not shown), strongly suggesting a defect specific for CXCR4.

As therapeutic IL-2 effectively restored membrane CXCR4 expression on CD4+ T-cells from 3 out of 4 patients (Figure 2B), we next investigated whether defective CXCR4-mediated chemotaxis could be normalized upon IL-2 treatment. To address this possibility, PBMC recovered from P6 immediately after a 5 days course of IL-2 treatment were tested for their ability to migrate towards CXCL12. As shown in Figure 3 (panels B and C), both CD4+ and CD8+ T-cell subsets displayed an increase in CXCL12-promoted chemotaxis. Overall, these findings indicate that the defect in membrane CXCR4 expression results in a loss of CXCR4 function in ICL T lymphocytes, which could be improved by IL-2 therapy.

Defective CXCR4 recycling in ICL CD4+ T-cells

Loss of membrane CXCR4 expression together with abnormal intracellular accumulation of CXCR4 and CXCL12 in ICL CD4+ T-cells suggested a defective intracellular routing of the
ligand/receptor complex. Processes involved in regulating CXCR4 activity include desensitization, internalization, degradation and recycling. To investigate the fate of CXCR4 upon CXCL12 exposure, we first set up the experimental conditions that allowed re-expression of CXCR4 at the surface of ICL CD4+ T-cells. After overnight culture of PBMC in complete medium supplemented with 10% FCS, CXCR4 surface expression reached comparable levels in ICL (geometric MFI range: 123 to 276) and control (geometric MFI range: 82 to 275) CD4+ T-cells. Of note, inter-individual variations in the steady-state levels of membrane CXCR4 expression in healthy donors have been documented. We then determined that the maximal CXCR4 endocytosis was reached at a concentration of 200 nM CXCL12 for control T-cells. Surface expression levels of CXCR4 in CD4+ T-cells from ICL (P1, P3 and P4) and healthy subjects (H1, H3 and H4) were then analyzed by flow cytometry. Following CXCL12 stimulation, the fraction of internalized CXCR4 (~60%) in P1 and P3 CD4+ T-cells was comparable to that detected in controls (Figure 4, white symbols, t40/0). In contrast, the rate of CXCR4 endocytosis was weaker (~30%) in CD4+ T-cells from P4 during the initial 40 min of CXCL12 treatment. Thus, CXCL12-promoted internalization of CXCR4 occurred normally in CD4+ T-cells from P1 and P3, whereas it was impaired in those from P4.

We then compared the fate of down-modulated CXCR4 in CD4+ T-cells from ICL and healthy individuals (t40/10 and above). In control cells, CXCR4 began to recycle to the cell surface immediately after agonist removal and reached 60-70% of pre-treatment levels within 2 hrs. In contrast, the recycling of CXCR4 in CD4+ T-cells from P1, P3 and P4 was defective, as we detected no surface re-expression of CXCR4 within the first 30 min of chase and no further increase in receptor density for up to 120 min. These findings provide evidence for defective CXCR4 recycling in ICL CD4+ T-cells. As therapeutic IL-2 effectively restored membrane CXCR4 levels in CD4+ T-cells from 3 out of 4 patients (Figure 2B), we next studied whether defective CXCR4 recycling could be normalized upon IL-2 treatment. To
address this possibility, PBMC from P3 and P4 incubated overnight with recombinant IL-2 or those recovered from P1 immediately after the course of IL-2 treatment were pulsed and chased with CXCL12. As shown in Figure 4 (black symbols), IL-2 exposure modulated neither CXCR4 endocytosis nor the kinetics of CXCR4 recycling in control and ICL CD4⁺ T-cells.

**CD4⁺ T-cell recovery during IL-2 treatment**

Four patients (P1, P2, P3 and P6) received several courses of recombinant IL-2 for up to 3 years. Treatment efficacy with respect to CD4⁺ T-lymphocyte recovery was variable, with one patient showing a sustained response (P2), two displaying transient responses (P1 and P6) and one not responding to treatment (P3). Results of immunophenotyping analyses during 4 courses of IL-2 administration are reported for P1 in Figure 5 and for all the 4 treated patients in Table 1. A progressive increase in total lymphocyte number, ascribed to an increase in CD3⁺ T-cells, was observed. Both CD4⁺ and CD8⁺ T-cell numbers rose transiently with each IL-2 cure. We did not observe a change in the distribution of naive and memory CD4⁺ T-cells following IL-2 therapy (data not shown). B-cell numbers did not vary significantly overtime, while a slow increase followed by a plateau phase was observed for NK cells. Five years later (i.e. after 42 IL-2 cycles), P1 is still requiring repeated cytokine administration every 12 weeks, as treatment withdrawal was associated with a drop of CD4⁺ T-cells (<100 cells/mm³) and reappearance of cryptococcal antigen in serum after sustained negativation for 48 months. After 3 years of IL-2 treatment, P2 presented a sustained CD4⁺ T-cell count recovery (498 cells/mm³) that was maintained one year after IL-2 treatment withdrawal. P6 received two cycles of IL-2 therapy in 2005 and 2008 with a sustained CD4⁺ T-cell recovery following the latest cycle (247 cells/mm³). In contrast, P3 did not exhibit any change in CD4⁺ T-cell count after IL-2 treatment and developed disseminated *M. kansasii* infection despite the
administration of 12 IL-2 courses (data not shown). It is noteworthy that P3 was the patient for which CXCR4 expression did not improve after *in vitro* addition of IL-2. These findings suggest an association between the capacity of IL-2 to restore membrane CXCR4 expression and its therapeutic effect on CD4+ T-cell homeostasis.

**DISCUSSION**

In the present study, we provide a novel insight into ICL pathogenesis based on the systematic investigation of six patients. We show that ICL, at least in all the patients included in our study, is associated with a profound defect of membrane CXCR4 expression in circulating naive and memory CD4+ T-cells, accompanied by an abnormal intracellular accumulation of CXCR4 and of its ligand CXCL12. We did not find evidence for mutations, increased plasma concentration of CXCL12, or changes in cytokine levels in ICL plasmas that could account for CXCR4 down-regulation. This defect seemed to be specific for CXCR4, since expression levels of CCR5 remained within the normal range or was increased depending on the patient. Reduced levels of membrane CXCR4 expression were associated with impaired chemotactic responses to CXCL12, but not to CXCL8, another α-chemokine. These findings suggest that ICL CD4+ T lymphocytes may be defective for CXCR4-specific functions *in vivo*, including homing, differentiation and recirculation within tissues. Loss of CXCR4 expression may also disturb T-cell priming within secondary lymphoid organs, as these cells may not encounter their cognate antigen in an environment that would allow efficient antigen presentation and activation. This issue may account for the limited proliferation and cytokine secretion capacity of residual ICL CD4+ T-cells, as confirmed here. Given that CXCR4 is the major cognate receptor for CXCL12 and that this interaction is critical for BM homeostasis, lymphocyte homing and recruitment into inflammatory sites (reviewed in21,23,46,47), we propose that defective CXCR4 expression contributes to
immunodeficiency during ICL. However, considering the variable immunophenotypes reported in ICL, we cannot exclude the possibility of a multi-factorial etiology of ICL.

CXCL12/CXCR4 interactions have proven a promising target to prevent undesirable cell activation or recruitment in different models of diseases. Disruption of CXCL12/CXCR4 interactions upon treatment with the selective CXCR4 antagonist AMD3100 leads to a rapid, transient and reversible mobilization of HPCs as well as mature granulocytes and lymphocytes in the peripheral blood of healthy mice and humans. Results from AMD3100 treatment suggest that CXCR4 blockade leads to an increase in circulating CD4+ T-cells. However, one should take into account that transient and chronic disruption of the CXCR4/CXCL12 axis appears to have distinct effects on T-cell homeostasis. Permanent intracellular retention of CXCR4 in the CXCL12-intrakine mouse model leads to a severe CD4+ T-cell lymphopenia, consistent with our findings in ICL. Thus, CXCR4 dysfunction in ICL may well account for the observed CD4+ T-cell lymphopenia.

The mechanism underlying the defect in membrane CXCR4 expression might involve impaired receptor recycling rather than synthesis. Early steps of CXCR4/CXCL12 interactions appear functional as physiologic CXCR4 endocytosis is preserved following CXCL12 binding in most ICL CD4+ T-cells. These findings suggest that, in ICL CD4+ T-cells, CXCR4-dependent signaling does occur and leads to phosphorylation events and β-arrestin recruitment required for receptor internalization (reviewed in ). In contrast, later steps in the intracellular routing process of the receptor appear defective in ICL CD4+ T-cells, as CXCR4 persists in intracellular compartments instead of being degraded and/or recycled back to the plasma membrane. The concomitant intracellular accumulation of CXCL12 strongly suggests that CXCR4 fails to dissociate from its cognate ligand. The molecular defect responsible for impaired CXCR4 recycling remains to be elucidated. It may vary according to patients. Indeed, both CXCR4 endocytosis and recycling were affected in CD4+
T-cells from P4, while only receptor recycling was impaired in those from P1 and P3. The CXCR4 defect appeared restricted to ICL T-cells, as membrane CXCR4 expression and function were preserved in monocytes, suggesting a T-cell-specific impairment in a gene product selectively involved in the regulation of CXCR4 recycling. Endopeptidases that degrade GPCR ligands within endosomes may play a role, since ligand dissociation is required before receptor recycling. Proteins that regulate CXCR4 vesicular trafficking and recycling (e.g. synaptotagmin 3 and PIM1) may also contribute to the defective CXCR4 expression in ICL T-cells.

The defective membrane CXCR4 expression in ICL T-cells is likely to perturb CD4+ T-cell homeostasis at several stages, including thymopoiesis. CXCR4 is believed to orchestrate the localization of early lymphoid progenitors to thymic regions, where lineage commitment and proliferation are controlled. Blockade of CXCR4 signaling causes an abnormal retention of thymocytes in the cortex. In mice reconstituted with progenitor cells expressing a CXCL12 intrakine, which prevents membrane CXCR4 expression, hematopoiesis is impaired in several compartments, including the thymus, and cell depletion is more pronounced for CD4+ than for CD8+ T-cells. These findings suggest that the CD4+ T-cell subpopulation is more sensitive to alterations in CXCR4 expression than its CD8+ counterpart. We observed functional defects in residual peripheral ICL CD4+ T-cells, which may reflect an activated and/or exhausted status of CD4+ T-cells that undergo prolonged homeostatic proliferation and are consistent with the reported increased apoptosis of ICL CD4+ T-cells.

From a therapeutic perspective, IL-2 showed efficacy at restoring membrane CXCR4 expression and function together with CD4+ T-cell counts in a subset of ICL patients. CXCR4 expression is reported to be inducible by cytokines of the γ-c family, including IL-2 and IL-7. Cytokine treatment, as well as triggering of certain surface receptors such as CD62L,
can cause the rapid relocalization of CXCR4 intracellular stores to the cell surface. In HIV-infected patients, intermittent IL-2 therapy is associated with an increased proliferation of both CD4+ and CD8+ T-cell populations and increased survival of both naive and central memory CD4+ T-cells. An expansion of naive CD25+ T-cells with some, but not all, characteristics of regulatory T-cells is also observed. Interestingly, IL-2 therapy enhances the number of circulating CD4+ T-cells that expressed high levels of CXCR4. In the present study, 3 out of 4 ICL patients responded to IL-2 therapy by an increase in CD4+ T-cell counts, with a sustained response in one case (P2) and transient responses in the two others (P1 and P6). Strikingly, increased CD4+ T-cell counts correlated with a recovery of membrane CXCR4 expression and function. Furthermore, the only patient (P3) who showed no improvement in CD4+ T-cell count upon therapy did not show membrane CXCR4 re-expression either after in vivo or in vitro IL-2 treatment. These data raise the possibility that in vitro analysis of CXCR4 induction on CD4+ T-cells by IL-2 might provide a useful screen to predict the patient’s responses to IL-2 immunotherapy. Overall, membrane CXCR4 expression was normalized on CD4+ T-cells from 5 out of the 6 patients after in vitro IL-2 stimulation, suggesting that IL-2 administration might have a beneficial effect in a significant fraction of ICL patients. This study, together with two previous case reports of IL-2 administration and one case report of IFN-γ treatment, provide a rationale for furthering cytokine-based immunotherapy in ICL patients. In conclusion, our data indicate that ICL is associated with a defect in membrane CXCR4 expression, which may be reversed by IL-2.
FOOTNOTES

We are grateful to patients who participated to the study. We thank the medical staff (A. Boibieux, J. Gaillat, B. Ponceau and M. Vidailhet) for their collaboration and referring their patients to us. We thank E. Cabannes (INSERM U819, Institut Pasteur, Paris, France) for help with genetic studies and are grateful to F. Baleux (Unité de Chimie Organique, Institut Pasteur, Paris) for providing us with the synthetic CXCL12. We thank V. Godié (INSERM U996, Université Paris-Sud 11, Clamart, France) for technical help and are grateful to D. Emilie (INSERM U996 and Service de Microbiologie-Immunologie Biologique, Hôpital A. Béclère, AP-HP, Clamart, France) for supporting funding application (AP-HP, grant number 07018). This work was supported by INSERM, Institut Pasteur, AP-HP and the European Union FP6 (INNOCHEM, grant number LSHB-CT-2005-518167).
AUTHORSHIP

DS-A designed research, performed research, contributed analytical tools, analyzed data and wrote the paper. KB designed research, performed research, contributed analytical tools, analyzed data and wrote the paper. LAC performed research, analyzed data and wrote the paper. LM performed research and analyzed data. FD designed research, analyzed data and wrote the paper. CD performed research. FA-S contributed analytical tools and analyzed data. OL initiated and coordinated the study, designed research, performed research, analyzed data, and was in charge of the writing committee. The authors have no conflict of interest to disclose.
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52. Matthys P, Hatse S, Vermeire K, et al. AMD3100, a potent and specific antagonist of the stromal cell-derived factor-1 chemokine receptor CXCR4, inhibits autoimmune


Table 1: Clinical and immunological profiles of six patients with ICL compared to healthy controls

<table>
<thead>
<tr>
<th>ICL patient #</th>
<th>Healthy controls</th>
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<tr>
<td>Age (yrs) at the time of study</td>
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<tr>
<td>43</td>
<td>51</td>
</tr>
<tr>
<td>42</td>
<td>50</td>
</tr>
</tbody>
</table>

Opportunistic infections

- *Cryptococcus neoformans*
  - disseminated meningeal bone
- *Alternaria sp.*
  - Skin (recurrent) skin + lung
- *Nocardia brasiliensis*
  - skin + lung
- *Mycobacterium tuberculosis*
  - Lung
- *Mycobacterium kansasii*
  - disseminated
- *Pneumocystis jirovecii*
  - Lung

<table>
<thead>
<tr>
<th>IgG (g/l)</th>
<th>7.9</th>
<th>12.0</th>
<th>7.5</th>
<th>12.0</th>
<th>11.1</th>
<th>6.2</th>
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<tbody>
<tr>
<td>IgM (g/l)</td>
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<td>0.6</td>
<td>1.6</td>
<td>1.7</td>
<td>1.1</td>
<td>0.4</td>
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<tr>
<td>IgA (g/l)</td>
<td>1.3</td>
<td>1.9</td>
<td>2.0</td>
<td>1.0</td>
<td>1.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Pre /post IL-2 values for

| Total lymphocytes (Ly/mm³) | 653 /857 | 390 /1340 | 165/200 | 799 / ND | 450 / ND | 979/990 | 1742 ± 429 |
| CD4⁺ T Ly/mm³ (%) | 20(31) /215(15) | 120(31) / 582 (43) | 6(4) /10(5) | 208(26) / ND | 185(41) / ND | 137(14)/247(25) | 44% ± 11 |
| CD8⁺ T Ly/mm³ (%) | 418(64) /377(44) | 148(38) /432(32) | 2(1%) / 5(2%) | 228(28) / ND | 81(18) / ND | 206(21)/178(18) | 28% ± 7 |
| B Ly/mm³ (%) | < 1 /<1 | 27(7) /ND | < 1 / < 1 | 40(5) / ND | 9(2) / ND | 176(18)/198(20) | 17% ± 9 |
| γδ T Ly/mm³ (%) | 144(22) /214(25) | 47(12)/ND | 46(28)/ND | 119(15) / ND | 71(15) / ND | 117(12)/40(4) | 3.5% ± 2 |
| NK Ly/mm³ (%) | 79(12) /215(15) | 63(16)/ND | 102(60)/ND | 144(18) / ND | 104(22) / ND | 343(35)/327(33) | 9.5% ± 4.8 |
Table 1 (Cont'd)

<table>
<thead>
<tr>
<th>CD4⁺ T Ly surface levels (%) of</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
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<td>&lt; 3</td>
<td>8</td>
<td>&lt; 3</td>
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<td>25 ± 5</td>
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<tr>
<td>CCR5</td>
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<td>8</td>
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<td>17</td>
<td>12</td>
<td>17.5</td>
<td>16 ± 8</td>
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<tr>
<td>CD8⁺ T Ly surface levels (%) of</td>
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<td></td>
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<tr>
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<td>9.7</td>
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<td>10.4</td>
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<td>19 ± 8</td>
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<tr>
<td>CCR5</td>
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<td>8</td>
<td>18</td>
<td>21</td>
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<td>22</td>
<td>12 ± 15</td>
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<tr>
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</tr>
<tr>
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<td>35</td>
<td>47</td>
<td>32</td>
<td>52</td>
<td>29</td>
<td>2.0 ± 0.6</td>
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<td>22</td>
<td>34</td>
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<td>0</td>
</tr>
<tr>
<td>CD8⁺ T Ly intracytoplasmic levels (%) of</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>9</td>
<td>12</td>
<td>&lt;1</td>
<td>2</td>
<td>8</td>
<td>3</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>CCR5</td>
<td>1</td>
<td>2</td>
<td>&lt;1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CXCL12</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

BM biopsies showed myelodysplasia and myelofibrosis in P3 and no abnormality in the 5 other patients. ND: not done.
**LEGEND TO THE FIGURES**

**Figure 1: Proliferative response and cytokine production by ICL PBMC.**
Top panels: Proliferation of CD4⁺ (left) or CD8⁺ (right) T-cells was assessed in the presence of *Staphylococcus aureus* Enterotoxin B (SEB), tetanus toxin (TT), candidin (CANDI), purified protein derivative from *Mycobacterium tuberculosis* (PPD) or the mannoprotein antigen from *Cryptococcus neoformans* (MANO) among PBMC from healthy (A) and ICL (B) subjects. Results are expressed as percentages of proliferating CD4⁺ and CD8⁺ T lymphocytes (means ± SEM and percentiles) after antigenic stimulation, as measured by the fraction of cells with decreased CFSE staining. Bottom panels: IFN-γ (left) or TNF-α (right) production by PBMCs from healthy (A) and ICL (B) individuals after *in vitro* stimulation. Supernatants were harvested 36 h after stimulation and measured for cytokine levels by the CBA method. Values represent means ± SEM and percentile of triplicate supernatant samples.

*P<0.001, as compared to control or ICL cells.

**Figure 2: Abnormal CXCR4 expression and intracellular detection of CXCL12 in ICL CD4⁺ T lymphocytes.** (A) Proportion of CD3⁺ CD4⁺ T-cells in whole blood sample from a representative ICL patient (P1) as determined by flow cytometry. (B) Membrane expression levels of endogenous CCR5 and CXCR4 in CD4⁺ T-cells from P1 (expressed as %). Whole blood recovered before (untreated) or after (*in vivo*) five days of therapeutic administration of IL-2 (4.5 millions units b.i.d. subcutaneously) or PBMC incubated overnight with 10 UI/ml of IL-2 (*in vitro*) were labeled using FITC-conjugated CCR5 and PE-conjugated CXCR4 mAbs. Background fluorescence was measured using the corresponding isotype control Ab. The inset summarizes the proportion of CXCR4⁺ CD4⁺ T-cells in the 3 patients (P1, P2, and P6) before and after IL-2 therapy. Mean CXCR4 expression is reported before and after the first 5
days cycle of IL-2 (p<0.001). (C) Intracellular staining of CXCL12 in CD4\(^{+}\)-gated T-cells from PBMC of an healthy (H1, white histograms) or ICL (P1, gray histograms) subject obtained before (untreated) or after (\textit{in vivo}) five days of IL-2 administration. Staining was done using an anti-human CXCL12 mAb followed by a FITC-conjugated anti-mouse Ab. The inset summarizes the proportion of CXCL12-containing CD4\(^{+}\) T-cells in the 3 patients before and after administration of the first cycle of IL-2.

**Figure 3: Impaired CXCL12-promoted chemotaxis of ICL T lymphocytes.** (A) Migration of PBMC from ICL patients (P1 and P2) and healthy subjects (H1 and H2) in response to 1 or 4 nM CXCL12. (B and C) Migration of PBMC from ICL patients (P1 and P6) and one healthy subject (H3) in response to serial dilutions of CXCL12 (0.1 to 30 nM) or to 30 nM CXCL8. PBMC from P6 were analyzed before (gray columns) or after (hatched columns) the course of IL-2 treatment. Transmigrated cells recovered in the lower chamber were counted by flow cytometry after gating on forward and side scatter to select lymphocytes (A) or after gating specifically CD4\(^{+}\) or CD8\(^{+}\) T lymphocytes (B and C) after staining with CD45, CD4, CD8 and CD3 mAbs. Results (medians \pm SD for triplicate wells) are expressed as the percentage of input lymphocytes (A), CD4\(^{+}\) (B) or CD8\(^{+}\) (C) T-cells that migrated to the lower chamber.

**Figure 4: CXCR4 endocytosis and recycling in ICL CD4\(^{+}\) T-cells.** PBMC from three independent healthy donors (H1, H3 and H4, white squares) and three ICL patients (P1, P3 and P4, white circles) were cultured overnight in complete medium supplemented with 10% FCS, allowing CXCR4 re-expression at the surface of ICL cells, then incubated for 40 min with 200 nM CXCL12 (Treatment, t\textsubscript{40}/0, CXCR4 endocytosis), and further cultured for up to 120 min in the absence of CXCL12 (Chase, t\textsubscript{40}/10 and above, CXCR4 recycling). The protein synthesis inhibitor CHX (50 \(\mu\)g/ml) was present throughout the experiment. Levels of
membrane CXCR4 expression were assessed by flow cytometry in CD3⁺ CD4⁺-gated T-cells. Effects of IL-2 on CXCR4 endocytosis and recycling were evaluated in CD4⁺ T-cells from healthy (black squares) and ICL (black circles) subjects. The kinetic of CXCR4 down-modulation in PBMC recovered from P1 immediately after the course of IL-2 treatment was compared to that obtained in cells from H1 recovered the same day and left untreated (gray squares). Displayed data are means of duplicate determinations and are expressed as percentages of CXCR4 expression (100% corresponding to CXCR4 expression at the surface of CD4⁺ T-cells incubated in medium alone).

Figure 5: CD4⁺ T-cell recovery in ICL patients upon therapeutic administration of IL-2. Changes in absolute leukocyte counts (A: lymphocytes, CD4⁺ T and B lymphocytes, and B: CD3⁺ and CD8⁺ T lymphocytes and NK cells) in one ICL patient (P1) during four courses of recombinant IL-2 treatment are plotted. Arrows indicate IL-2 administration.
Figure 3

Graph A: Percentage of PBMC input over CXCL12 (nM) for P1, P2, H1, and H2.

Graph B: Percentage of CMI+ T cell input for P1, P6, P6-IL2, H3, and H3.

Graph C: Percentage of CD3+ T cell input for P1, P6, P6-IL2, H3, and H3.
Figure 5

(A) Lymphocytes/mm³

(B) CD3⁺ or CD8⁺ T cells/mm³

CD4⁺ T or B cells/mm³

NK cells/mm³

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Idiopathic CD4+ T-cell lymphocytopenia is associated with impaired membrane expression of the chemokine receptor CXCR4

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