Injection of Glycosylated Recombinant Simian IL-7 Provokes Rapid and Massive T-cell Homing in Rhesus Macaques

Stéphanie Beq 1, Sandra Rozlan 2*, David Gautier 1*, Raphaëlle Parker 1, Véronique Mersseman 1, Clémentine Schilte 1, Brigitte Assouline 2, Iann Rancé 2, Pascal Lavedan 3, Michel Morre 2 and Rémi Cheynier 1

1. Département de Virologie, Institut Pasteur, 25-28, Rue du Docteur Roux 75724 Paris Cedex 15, France
2. Cytheris S.A., Technopolis, 175 rue J.J. Rousseau, 92130 Issy les Moulineaux, France
3. Animalerie Centrale, Institut Pasteur, 25-28, Rue du Docteur Roux 75724 Paris Cedex 15, France

* S.R. and D.G. equally contributed to this work

Running title: IL-7 induces massive T-cell homing into organs

Corresponding author:
Rémi Cheynier
Département de Virologie
Institut Pasteur
25-28, Rue du Docteur Roux 75724 Paris Cedex 15, France.
Tel; (33)1 45 68 87 71
Email: remi.cheynier@pasteur.fr
ABSTRACT
Interleukin-7 (IL-7), the principal cytokine implicated in thymopoiesis and peripheral T-cell homeostasis, is presently under evaluation in human diseases characterized by persistent lymphopenia. Unexpectedly, prior to the eventual IL-7-driven T-cell expansion, all treated patients show a profound T-cell depletion 24 hours following injection. The current study utilizes the Rhesus macaque model to investigate the mechanisms involved in this IL-7-induced T-cell depletion. We identify a new critical function of IL-7 that induces massive and rapid T-cell migration from the blood into various organs, including lymph nodes, parts of the intestine and the skin. This homing process was initiated following the induction of chemokine receptor expression by circulating T-cells and the production of corresponding chemokines in target organs. Finally, we demonstrate that the IL-7-induced cell cycling is initiated within these organs before T-cells migrate back into the blood stream, indicating that T-cell homing is required for in vivo IL-7 function.
INTRODUCTION

Interleukin 7 (IL-7), the cytokine that promotes precursor B-and T-cell maturation\textsuperscript{1-3} and sustains peripheral T-cell homeostasis\textsuperscript{4-6}, is currently in phase I-II trials in diseases characterized by persistent lymphopenia. Despite the fact that interim results from these investigations tentatively support the efficacy of this molecule in producing long term T-cell increases in patients, an unexpectedly strong T-cell depletion was observed in all treated patients during Day 1 of therapy (\textsuperscript{7} and Irini Sereti, personal communication). Similar transient T-cell depletion was previously observed in both CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell subsets in IL-2-treated HIV-1 infected patients. In this study, massive apoptosis was observed at the end of the IL-2 cycle coinciding with the peak of proliferation\textsuperscript{8}. While apoptosis is not observed under physiological concentrations of IL-7 in culture, during the first hours following IL-7 injection in patients the pharmacological IL-7 plasma concentration may result in a different outcome. Otherwise, T-cell depletion may also be the consequence of a massive T-cell redistribution.

Various chemokine-chemokine receptor couples participate in specific T-cell homing into both lymphoid and non-lymphoid organs\textsuperscript{9}. In particular, CCL19-CCR7, CCL21-CCR7 and CXCL12-CXCR4 are primarily implicated in recruiting T-cells into secondary lymphoid organs\textsuperscript{10,11}, while CCL20-CCR6 interaction drives T-cells to the gut\textsuperscript{12} and CCL25 allows CCR9-expressing T-cells to home to the small bowel\textsuperscript{12,13}. Engagement of the chemokines with their receptors on the leukocyte membranes triggers intracellular signals resulting in tight adhesion of the migrating cells through integrins, extravasation from the blood stream into tissues along chemokine gradients, eventually allowing cell activation. Interestingly, \textit{in vitro} IL-7 stimulation was shown to induce CXCR4 expression by naive T-cells, suggesting potential homing consequences\textsuperscript{14}.

IL-7 is constitutively produced by stromal and epithelial cells in the bone marrow\textsuperscript{15}, in the thymus\textsuperscript{16}, and in lymph nodes\textsuperscript{6,17-19} but also in the skin\textsuperscript{20} and mucosal tissues\textsuperscript{21}. Its affinity for heparan sulfate proteoglycans (HSPGs) on the stromal cells\textsuperscript{22,23} suggests that a higher local IL-7 concentration in productive tissues than in plasma may play a role in its functions.
Contrary to the situation in the thymus, where IL-7 interaction with HSPG does not seem required \(^{24}\), HSPGs provide a docking site for IL-7 in the bone marrow, controlling its availability to B-cell precursors \(^{23}\). In the other sites of IL-7 production, the implications of local IL-7 concentration on its function remain to be clarified.

After confirming in the healthy Rhesus macaque model that the injection of recombinant glycosylated simian IL-7 (R-sIL-7gly) is, as in human patients, immediately followed by massive depletion of circulating T-cells, we investigated the consequences of R-sIL-7gly injection on both T-cell apoptosis and T-cell homing.

**MATERIALS AND METHODS**

**Animal care and treatment**

The Rhesus macaques included in this study were handled in accordance with European guidelines for care and treatment of research animals and the experiments performed on them were approved by the ethics committee of the Institut Pasteur. The animals were seronegative for SIVmac, simian T-cell leukemia virus type 1, simian retrovirus type 1 (type D retrovirus), and herpes virus B. For all injections and blood draws, the animals were anesthetized with ketamine (Imalgène 1000, Merial, Lyon, France).

In a first phase of the experiment, two healthy Rhesus macaques of Chinese origin (#14424 and #14694) and an animal (#17021) previously immunized against simian IL-7 received a single subcutaneous injection of recombinant glycosylated simian IL-7 (R-sIL-7gly; 80µg/Kg of body weight), a dose previously shown to stimulate T-cell expansion in both healthy and SIV-infected macaques \(^{25,26}\). Macaque #17021, extensively immunized with 3 injections of non-glycosylated recombinant simian IL-7 and complete Freund adjuvant, demonstrated significant levels of anti-IL-7 neutralizing antibodies (>1/2500) at the time of the experiment.
Following R-sIL-7gly injection, blood samples (3ml on EDTA) were drawn from both treated animals at 6, 12, 48 and 96 hours, as well as at Day 7 and Day 14. In addition, two non-injected animals (#21045 and #26007) served as controls and were subjected to the same sampling protocol. Four months later, when all the measured parameters had returned to baseline levels, macaques #14424 and #14694 received a second injection of R-sIL-7gly. Macaques #26007 and #21045 served as controls. Blood samples were drawn at 6, 12, 48 and 96 hours (H0 to H96), as well as at Day 7. Moreover, at H0, H6 and H24, axillary or inguinal lymph nodes were surgically removed from macaques #14424, 14694 and 26007 at H0, H6 and H24.

In a second phase of the experiment, 3 healthy Rhesus macaques were subcutaneously injected with R-sIL-7gly (80µg/Kg of body weight) and sacrificed at Day 1 (#26007-2 and #41127-2) and Day 7 (#40885-2). A fourth animal (#21045-2) without R-sIL-7gly injection was sacrificed as a negative control. For this experiment, we also used historical samples from a healthy macaque, kindly provided by Dr Cécile Butor of the Institut Cochin, Département d'Immunologie, INSERM U567, CNRS UMR8104, Université Paris-Descartes, Faculté de Médecine, Paris and Université Paris 7, Denis Diderot, France .

**Immunophenotyping and flow cytometry analysis.**

For FACS analysis performed on total blood samples, (EDTA)-treated blood cells were incubated for 15 minutes with conjugated monoclonal antibodies (mAbs). For intracellular labeling, cells were permeabilized with the Cytofix/Cytoperm Kit (Becton Dickinson) before incubation with specific mAbs following the manufacturer’s instructions. Erythrocytes were lysed with the Beckman Coulter Lysing Kit according to the manufacturer’s instructions. Samples were then washed and fixed in 2% paraformaldehyde phosphate-buffered saline (PBS, PFA 2%). Immunostainings were produced using a Cyan cytofluorometer (Dako Trappes, France) and analyzed with FlowJo 8.7 software.
For FACS analysis of tissue samples, cell suspensions were obtained by scraping and mild enzymatic dissociation in PBS containing 20U/mL collagenase VII (Sigma, St Quentin Fallavier, France) and 40U/mL DNase I (Sigma) in 2% fetal calf serum for 30 minutes at 37°C.

The different monoclonal antibodies used in this study were: CD3-APC-Cy7, CD4- PercP-Cy5.5, CD28-PE, CD95-APC purchased from Becton-Dickinson (BD, Le-Pont-de-Claix, France); CD8-PE-Cy7, CD31-biotin, streptavidin-PE-Texas-RED and CD31-DY590 (proteogenix-Oberhausbergen, France); Ki67- fluorescein isothiocyanate (FITC) and Bcl-2-FITC from Dako (Trappes, France); CCR7-FITC, CCR6-FITC, CCR9-FITC and CXCR4-FITC (R&D-Lille, france); caspase 8 and caspase 9 (ABCAM-Paris, France).

**Immunohistochemistry**

Snap-freezing was performed using Cryomold (Tissue-Tek, Labonord-Templemars, France) and optimal cutting temperature compound (OCT Tissue-Tek). The cups were placed in isopentane and cooled in liquid nitrogen. Samples were then stored at –80°C until used.

Frozen tissue sections were utilized for immunohistochemical detection of CD3, CD127 and Ki67 molecules. Briefly, frozen tissue sections with a thickness of 4µm were prepared and mounted on glass slides. The sections were then air dried for 1 hour and fixed with acetone for 10 min. Prior to immunolabelling, the sections were fixed with acetone/methanol (50/50) for 5 min at room temperature (RT) and then re-hydrated with TBS pH 7.6 (Dako, Trappes, France) for 5 min at RT. Endogenous peroxydase activity was blocked with hydrogen peroxide 3% for 5 min at RT. To reduce non-specific background binding of the secondary antisera, sections were incubated for 20 min with rabbit serum. Sections were then incubated for 1h with primary antibody and subsequently incubated with biotinylated rabbit anti-mouse immunoglobulin (Dako, Trappes, France) for 30 min, followed by streptavidin biotin peroxydase complex for an additional 30 min. Each incubation was followed by a PBS wash.
To facilitate visualization of the reaction, DAB was added to the sections which were then counterstained with Mayer’s hematoxilin and mounted under Shandon synthetic montant (Thermo Fischer Scientific, Cergy Pontoise, France). Slides were examined using an Eclipse E800 microscope and a DXM1200 digital camera (Nikon, Champigny sur Marne, France). Images were acquired using ACT-1 software (Nikon).

**Chemokine quantification**

Primers specific for each chemokine (CCL19, CCL20, CCL21, CCL25) and the HPRT gene (used as a housekeeping gene) were based on macaque cDNA sequences (accession numbers: ENSG00000172724, ENSG00000115009, ENSG00000137077, ENSG00000131142, ENSG00000165704). The oligonucleotides used for chemokine quantifications are shown in table 1. PCR products obtained with the outer primer pairs for each chemokine were cloned, together with the out HPRT amplicon, into a Topo cloning vector™ (Invitrogen, Cergy-Pontoise, France) and utilized for the generation of the standard curves. Parallel quantification of each chemokine and the HPRT was performed for each sample using LightCycler™ technology (Roche Diagnostics). Briefly, tissue samples (1mm³) were dissociated and mRNA was extracted using an RNeasy kit (Qiagen, Courtaboeuf, France). Following a reverse transcription step, cDNAs were PCR amplified in a final volume of 100 µl (10 min initial denaturation at 95°C, then 22 cycles of 30 sec at 95°C, 30 sec at 60°C, and 2 min at 72°C using outer 3’/5’ primer pairs). Each chemokine was co-amplified together with HPRT sequences for each sample. Chemokines and HPRT were then quantified on each of these PCR products in LightCycler™ experiments performed on 1/100th of the initial PCR products. PCR conditions were the following: 1 minute initial denaturation at 95°C, followed by 40 cycles of 1 sec at 95°C, 10 sec at 60°C, and 15 sec at 72°C with inner primers; fluorescence measurements were performed at the end of elongation steps. Chemokines and
HPRT quantifications were performed in independent experiments using the same first-round serial dilution standard curve. The chemokine and HPRT were quantified in triplicate for all samples studied. Chemokine mRNA concentration was normalized to HPRT mRNA in each sample. Since baseline chemokine expression levels might be different between animals, the results are presented as a fold expression over a reference organ chosen to present low and stable expression for the given chemokine in the four animals (i.e. jejunum for CCL19, skin for CCL20 and lung for both CCL21 and CCL25)\textsuperscript{27}.

**Plasma cytokine quantification**

Cytokines in the plasma were quantified using the human 27-Plex\textsuperscript{TM} and 23-Plex\textsuperscript{TM} kits (Biorad, Marnes-la-Coquette, France) following the manufacturer’s instructions. Quantifications were performed in quadruplicate on 50µl of plasma. Out of the 50 molecules detected by these kits, 25 cross-reacted with their simian orthologs (IL-1\textbeta, IL-1RA, IL-5, IL-10, IL-12, IL-13, IL-16, MCP-1, Eotaxin, GCSF, IFN\alpha, IFN\gamma, CCL3, CCL4, CCL-5, CCL-7, CCL-27, CXCL8, CXCL9, MIF-3, NGF\beta, SDF-1, SCF, TRAIL and SCGF\beta). Baseline values for these molecules in Rhesus macaques were on the same order of magnitude as their human counterparts. Results obtained on a BioPlex\textsuperscript{TM} instrument were analyzed with BioPlex\textsuperscript{TM} software.

**Statistical analysis**

Statistical analyses (Mann-Whitney and paired Student T-tests) were performed using the VassarStats website (http://faculty.vassar.edu/lowry/VassarStats.html) and the StatView F-4.5 statistical software package (Abacus Concepts).
RESULTS

Injection of R-sIL-7gly leads to an immediate drop in all peripheral T-cell subsets

Five healthy Rhesus macaques (#14424, #14694, #26007-2 #41127-2 and #40885-2) were subcutaneously inoculated with 80µg/Kg of body weight of R-sIL-7gly as described in the materials and methods section of this paper. As observed in IL-7 treated patients, all R-sIL-7gly-treated animals demonstrated a strong peripheral lymphopenia during the first day following injection (Fig.1A) 25,28. Notably, despite the fact that T-cell increase was not observed at Day 7 in macaque #40885 (designated a poor responder), the initial decrease in lymphocyte counts was also observed in this animal. In contrast, non-injected control animals (#21045 and #26007) sampled on the same schedule as the treated monkeys did not show a significant change in their circulating lymphocyte counts (Fig.1A, right panel, open symbols).

More importantly, in an animal extensively immunized against non-glycosylated recombinant simian IL-7 (#17021) and presenting high titers of anti-R-sIL-7 neutralizing antibodies (>1/2500), injection of R-sIL-7gly did not lead to any substantial change in lymphocyte counts (Fig.1A, right panel; black diamonds). Four months later, when all the measured parameters had returned to baseline levels, a second injection of R-sIL-7gly given to #14424 and #14694 led to a similar drop in circulating lymphocytes (supplemental data 1).

Most of the observed lymphocyte decline occurred in T-cell subsets. Indeed, up to 85% of the CD4+ and CD8+ T-cells had disappeared from circulating blood by 6 hours following R-sIL-7gly injection (p<0.01 or p<0.05 in both CD4+ and CD8+ at H6, H12, H24 and H48; Fig1B and 1C.). In contrast, untreated control monkeys and the immunized animal demonstrated less than a 20% decline in circulating CD4+ and CD8+ T-cell counts (Fig.1B and 1C, right panels, open symbols and black diamonds), ruling out a consequence resulting from repeated blood sampling. This demonstrates that T-cell depletion was a direct consequence of the R-sIL-7gly
injection. In all treated monkeys, B-cell counts were much less affected by the R-sIL-7gly treatment (Fig. 1D).

We next analyzed the evolution of naive, central memory and effector memory T-cells as defined by CD95 and CD28 expression in CD4+ and CD8+ T cells. In Rhesus macaques, all T-cell subsets express the IL-7 receptor α chain (CD127), likely explaining their sensitivity to IL-7. The CD4+ and CD8+ T-cell drop that occurs during the first day after R-sIL-7 injection is a consequence of naive (CD95-CD28+), TCM (CD95+CD28+) and TEM (CD95+CD28-) T-cell depletion (Fig. 1E).

Two non-exclusive hypotheses could explain such a drastic and sudden drop in circulating T-cell counts: (1) either lymphopenia was a consequence of massive cell death induced by pharmacological plasma cytokine concentration; and/or, (2) the injection of R-sIL-7gly induced T-cell migration out of the blood.

R-sIL-7gly injection does not induce T-cell apoptosis

We first explored T-cell apoptosis in the blood (sampled at H0, H6 and Day 4 after R-sIL-7gly injection) of macaques #14424 and #14694. The active form of either caspase 3, caspase 8 or caspase 9 (supplemental data 2) remained barely detectable in the tested samples, demonstrating that programmed cell death cannot account for the observed massive depletion of circulating T-cells. Moreover, the distribution of CD4+ and CD8+ T-cells in the axillary lymph nodes remained unchanged throughout the first days following R-sIL-7gly injection while CD127 expression was strongly down modulated (supplemental data 2). Again, caspase 3, 8 and 9 activation as well as DNA fragmentation remained negative in these organs (supplemental data 2). This again shows that despite good tissue distribution in the lymph nodes, R-sIL-7gly did not induce massive T-cell apoptosis in the secondary lymphoid organs.
The absence of apoptosis in both lymph nodes and peripheral blood suggests that R-sIL-7gly-induced peripheral lymphopenia might be a consequence of T-cell redistribution from blood to organs. Since many lymphoid and non-lymphoid organs represent the usual homing sites for T lymphocytes, we further investigated the expression of molecules potentially involved in T-cell migration.

*In vivo R-sIL-7gly-stimulated circulating T-cells over-express chemokine receptors*

We investigated the expression of chemokine receptors implicated in T-cell homing into secondary lymphoid organs (CCR7 and CXCR4), the colon (CCR6) and the small bowel (CCR9), the principal homing organs targeted by T lymphocytes. As observed following *in vitro* IL-7 stimulation 14, a strong increase of CXCR4 expression was observed in the CD4+ and, to a lesser extent, the CD8+ subsets (Fig. 2A, top panel).

In contrast, we did not observe significant modification of the expression level of CCR7 in the treated monkeys. However, in healthy Rhesus macaques the vast majority of circulating T-cells expressed significant levels of CCR7 (70-90% in CD4+ T-cells and 25-50% in CD8+ T-cells; Fig. 2A, second panel), suggesting that CCR7-mediated T-cell homing is indeed possible and depends only upon the expression of CCR7 ligands (CCL19 and CCL21).

Finally, by 48 hours following R-sIL-7gly treatment a 1.5- to 2-fold increase of CCR6-expressing cell frequency was observed in both CD4+ and CD8+ subsets. In contrast, CCR9 expressing cell frequency was only increased in CD4+ T-cells (23-35% at Day 2 as compared to 8-10% at baseline) while remaining unchanged in CD8+ T-cells. Both CCR6 and CCR9 over expression were maintained for at least 4 days (Fig. 2A, bottom panels).

CCR6, CCR7, CCR9 and CXCR4 expression remained stable over time in both the non-injected monkeys and the immunized animal that received R-sIL-7gly injection (data not shown).
These data demonstrate that R-sIL-7gly injection immediately triggers chemokine receptor expression by circulating T-cells, allowing T-cell redistribution.

The analysis of chemokine receptor expression in the various T-cell subsets showed that these cells respond differently to R-sIL-7gly stimulation by expressing a particular set of chemokine receptors (Fig. 2B). CD4+ TCM demonstrated a major increase of CCR6, CCR9 and/or CXCR4 expression while CCR7 expression remained high in this subset (Fig. 2B). Similarly CXCR4, CCR6 and CCR9 were over-expressed by naive and TEM CD4+ T-cells (Fig. 2B). In the CD8+ subsets, R-sIL-7gly treatment induces the over-expression of both CXCR4 and CCR6 in TCM and only CXCR4 in naive T-cells. In these 2 subsets, CCR7 expression was reduced upon treatment (Fig. 2B). Finally, the expression of the four receptors in the TEM CD8+ subset remained low (Fig. 2B).

Chemokines implicated in T-cell homing are locally produced in organs

To precisely analyze the different organs that may receive the migrating T-cells, macaques #26007-2 and #41127-2 were sacrificed 24 hours after IL-7 treatment (i.e. at the peak of T-cell depletion) and animal #40885-2 at Day 7 post-injection. At autopsy, axillary, inguinal and mesenteric lymph nodes, ileum, jejunum, colon, rectum as well as skin and lungs were sampled. Similar samples were taken from an animal sacrificed without R-sIL-7gly injection (#21045-2). Messenger RNAs coding for CCL19 (CCR7 ligand), CCL20 (CCR6 ligand), CCL21 (CCR7 ligand) and CCL25 (CCR9 ligand) were quantified in several small pieces (±1mm³) from all tissue samples (Fig. 3).

CCL21 mRNA levels were increased in the lymph nodes of macaques sacrificed at Day 1 and at Day 7 (13- and 14-fold over baseline values respectively, p=0.0004 and p=0.003, Fig. 3). CCL19 expression was also elevated in the lymph nodes at Day 7 (12-fold increase over baseline, p=0.08). Of note, similar chemokine expression was observed in inguinal, axillary and mesenteric lymph nodes. Moreover, CCL19 was preferentially over-expressed in the
rectum (14- and 19-fold over baseline at Day 1 and 7, p=0.03, Fig. 3) and in the lungs (9- and 157-fold increase, p=0.02; Fig. 3 bottom panels), while CCL21 was found over-expressed in the skin (59- and 28-fold over baseline at Day 1 and Day 7 respectively, p=0.02; Fig. 3 bottom panels) and in the jejunum (58- and 28-fold, p=0.06 and p=0.034; Fig. 3 bottom panels). In contrast, these chemokines remained at baseline levels in the other tested organs.

Similarly, CCL25 mRNA concentration was increased in the ileum and jejunum (9- and 29-fold at Day 1 and Day 7 in the ileum, p=0.034 and p=0.014; 7- and 54-fold in the jejunum, p=0.06 and p=0.02; Fig. 3).

Finally, CCL20 mRNA concentration rose in the colon and rectum (83- and 151-fold at Day 1 and Day 7 in the colon, p=0.016 and p=0.014; 3- and 5-fold in the rectum, p=0.027 at Day 7; Fig. 3).

These data demonstrate that the classic T-cell homing sites were responding to R-sIL-7gly injection by the induction of organ-specific chemokine mRNA production, again suggesting T-cell homing into these different organs.

R-sIL-7gly injection leads to the expression of various molecules implicated in T-cell trafficking

Using the human Bioplex™ assay, we next evaluated, the evolution of plasma concentration of various molecules implicated in T-cell trafficking and inflammation. Out of the 50 molecules screened by the 27-Plex™ and 23-Plex™ kits, 25 cross-reacted with their simian orthologs (see materials and methods section). A transient rise of plasma concentration of CXCL12 (1.5-fold increase at H96), MIF (3- to 6-fold), CCL3 (3- to 4-fold increase at H6) and CCL4 (1.5- to 2-fold) - molecules known to participate in T-cell homing into lymphoid organs 30,31 - was observed in both treated macaques (Fig. 4A). Similarly, some molecules implicated in T-cell homing into non-lymphoid organs were also over-expressed following
IL-7 treatment: CXCL9 (1.5- to 2-fold) and CXCL8 (5- to 10-fold) \(^{32,33}\) (Fig. 4B). Finally, plasma concentrations of IL-1beta and IL-1RA, molecules implicated in transendothelial migration \(^{34}\), were augmented after R-sIL-7gly injection (10- to 20-fold and 10- to 100-fold, respectively; Fig. 4C).

These data demonstrate that in addition to chemokine and chemokine receptor expression, R-sIL-7gly injection leads to significant and reproducible \textit{in vivo} production of various molecules involved in T-cell trafficking.

\textit{Circulating T-cells home to various peripheral organs}

In order to confirm that R-sIL-7gly injection effectively triggers T-cell homing to the lymph nodes, gut and skin, tissue samples from animals #26007-2, #41127-2 (sacrificed 24 hours after R-sIL-7gly injection), #40885-2 (sacrificed at Day 7) and #21045-2 (non-injected animal) were subjected to immunohistological labeling with anti-CD3 monoclonal antibodies (Fig. 5). T-cell infiltration was observed in the skin and the \textit{lamina propria} of the ileum, the colon and the rectum (Fig. 5A to 5F). Quantifying CD3+ T-cells in 7 to 10 fields (0.09 mm\(^2\) each) randomly selected from four slides for each organ confirmed that the number of CD3+ T-cells per field was significantly increased by Day 1 in the skin (p=0.001; Fig. 5A right panel), the ileum (p=0.003; Fig. 5D right Panel), the colon (p=0.018; Fig. 5E right Panel) and the rectum (p=0.05; Fig. 5F right Panel). In all organs but the colon, T-cell numbers remained significantly higher at Day 7 as compared to the control animal. In contrast, the density of CD3+ T-cells was not significantly modified in the lymph nodes.

These data confirmed that R-sIL-7gly induces T-cell homing into various non-lymphoid organs including the \textit{lamina propria} of several parts of the gut (ileum, colon and rectum) and skin. Moreover, the expression of CCR7 and CXCR4 on circulating T-cells (Fig. 2), the increase of CXCL12 plasma concentration (Fig. 4) and the production of CCL19 and/or CCL21 mRNA in lymph nodes (Fig. 3) also suggest homing into secondary lymphoid organs.
Similarly, the production of CCL19 in the ileum and the rectum and that of CCL21 in the jejunum (Fig. 3) suggest that R-sIL-7gly injection also triggers T-cell migration into the lymphoid follicles of the gut.

**T-cells start cycling in the organs before migrating back into peripheral blood**

Following the initial decrease in circulating T-cell numbers, we observed a rebound in T-cell counts starting at Day 4. In all T-cell subsets, the gain represents 50% to 150% of the initial circulating T-cell counts at Day 7 (Fig. 1 and supplemental data 1). At that time, repopulating T-cells were indeed cycling as demonstrated by the frequency of Ki-67+ T-cells (20-30% and up to 60% at Day 7 in the CD4+ and CD8+ T-cell subset, respectively; Fig. 6A). In contrast, during the first two days following R-sIL-7gly injection, when circulating T-cells were over-expressing chemokine receptors as well as the anti-apoptotic molecule Bcl-2 (Fig. 6B), T-cell cycling was not yet evidenced in circulating blood. Interestingly, in the lymphoid tissue (LN and intestinal follicles) in the lamina propria of the ileum, the colon and the rectum as well as in the skin, a statistically significant increase of Ki-67 expression was observed as early as one day following R-sIL-7gly injection (p<0.05; Fig. 6C and 6D).

This demonstrates that T-cell cycling is initiated as early as one day following IL-7 stimulation within the organs where the injected cytokine induced their migration. In contrast, the cells remaining in the blood during the first days of IL-7 stimulation were not proliferating. Cycling T-cells eventually returned to the blood by Day 4, leading to increased numbers of circulating T-cells in the treated monkeys.

**DISCUSSION**

In the last few years, therapeutic stimulation of the immune system has begun to have an increasingly important impact on medical practice. In particular, IL-2 and IL-7 are currently
under clinical investigation for treatment of severe lymphopenia and have produced promising results. The significant increase of circulating T-cells observed in most IL-2-treated HIV-1 infected patients is the net result of the increase of both proliferation and apoptosis. Consequently, such a treatment, even at its best, does not restore, and in fact probably reduces, T-cell repertoire diversity. Similarly, in the first patients treated with IL-7, the observed initial T-cell depletion could influence the efficacy of this treatment in restoring T-cell repertoire diversity. However, we have herein demonstrated that the initial T-cell depletion observed during R-sIL-7gly treatment is not due to massive T-cell apoptosis but is instead a consequence of massive cell redistribution.

The rapid T-cell redistribution observed in IL-7 treated macaques suggests that IL-7 concentration in the target organs is important for T-cell function. Indeed, higher IL-7 concentration is necessary to trigger in vitro T-cell cycling than to stimulate cell survival. In vivo, higher local concentration might be achieved in organs where the cytokine is trapped on heparan sulfates at the stromal cell surface. Indeed, in preliminary experiments the injection of radiolabelled IL-7 in cynomolgus macaques led to a higher concentration in lymph nodes and spleen (20.8 to 32.8 ng/g of tissue), in the intestine (16.5, 19.1 and 21.2 ng/g in the bowel, colon and rectum, respectively) and in the lungs (18.7 ng/g) than in the blood (3.9 ng/g).

Differential expression of chemokine receptors by these different T-cell subsets (Fig. 2) as well as the chemokine production in non-lymphoid tissues such as the skin, lungs and intestine (Fig. 3), suggests a particular function for T-cell homing in these tissues. Indeed, IL-7 participates in T-cell responses to antigenic stimulation. Moreover, most of the molecules that were induced following IL-7 treatment were described as participating in T-cell homing into these organs during inflammatory reactions. The expression patterns of chemokines/chemokine receptors suggest that naive and memory CD4+ T-cells are directed to the gut through CCR6/CCL20 and/or CCR9/CCL25 expression while all T-cell subsets (but
the TEM CD8+ that barely express CCR7) might migrate into the skin and the lungs where CCR7-ligands (CCL19, CCL21) expression is increased. The involvement of IL-7-driven T-cell homing into non-lymphoid tissues in innate and adaptive immunity remains to be clarified but it is possible that the injection of R-sIL-7gly mimicked certain aspects of antigen induced immune activation by initiating T-cell migration to the sites of frequent pathogen intrusion.

Both \textit{in vitro} and \textit{in vivo}, IL-7 induces CXCR4 up regulation by T lymphocytes, suggesting a role of IL-7 in T-cell homing into CXCL12-rich tissues. Indeed, our \textit{in vivo} experiments showed that IL-7-induced CXCR4 over-expression coincides with an increased CXCL12 plasma concentration (Fig. 2 and 4). In addition, we showed that R-sIL-7gly injection stimulates CCL19 and CCL21 production by secondary lymphoid organs in which all T-cell subsets could thus be recruited through constitutive CCR7 expression (Fig. 2 and 3). Despite the fact that homing into lymph nodes is difficult to demonstrate in the macaque model, these data confirm observations made in mice, demonstrating the importance of lymph nodes in the regulation of naive T-cell homeostasis through IL-7 and CCL19 production\textsuperscript{6}, but also through CXCR4/CXCL12 expression. Finally, the fact that at Day 1 post R-sIL-7gly injection, T-cell cycling occurs in the lymph nodes and intestinal follicles while it remains undetectable in blood further reinforces this conclusion and suggests that homing to these organs is crucial for complete \textit{in vivo} IL-7 stimulation (Fig. 6C).

The local chemokine production in the different target organs suggests that resident cells are responsive to IL-7. Interestingly, monocytes and dendritic cells were shown to be essential for IL-7 induced proliferation\textsuperscript{43,44} and, together with fibroblasts, to produce most of the proteins observed in R-sIL-7gly-treated macaques\textsuperscript{44-47}. Further investigation of the impact of IL-7 on the production of these molecules by cells located in tissues will help in understanding both homeostatic and inflammatory homing processes induced by IL-7.
In summary, we have identified a novel property of IL-7 that when injected to healthy Rhesus macaques, rapidly initiates massive T-cell homing into secondary lymphoid organs and various epithelia. This effect is a direct consequence of both an increased expression of chemokine receptors (CXCR4, CCR6 and CCR9) by circulating T-cells and the initiation of chemokine transcription in the target organs. Homing processes were facilitated by the IL-7-dependant production of various molecules implicated in transendothelial cell migrations (IL-1β, IL-1RA, CCL3, and CCL4). However, other chemokine/chemokine receptors as well as other molecules implicated in T-cell trafficking can also be involved in the IL-7-induced cell migrations, in particular in the CD8+ subsets. By targeting circulating T-cells at the natural sites of IL-7 production, subsequently leading to T-cell proliferation, IL-7-induced T-cell homing appears to play an important role in the homeostatic regulation of peripheral T-cell pools. As previously observed, T-cell proliferation eventually leads to a 1.5 to 2.5-fold increase in circulating T-cells a week after IL-7 treatment. Interestingly, the IL-7-induced homing process drove circulating T-cells to different organs, including several parts of the gut where massive T-cell proliferation subsequently occurs.

Considering the results presented here and their possible impact on the treatment of HIV-1 infected patients, it would appear to be of great importance to evaluate the potential of IL-7, in combination with HAART in stimulating the T-cell repopulation of the gut, known to be massively T-cell depleted early in the course of HIV infection and thereby opening the patient to the effects of opportunistic infections and malignancies which are frequently associated with a weakened immune system in this patient population.

**Acknowledgments**

This work was supported by Institut Pasteur, Cytheris S.A. and the ANRS (Agence Nationale de Recherches sur le SIDA et les hepatites virales). This work was carried out in partial
fulfillment of R.P. doctoral thesis at the Université Paris 6 and D.G. doctoral thesis at the Université Paris 7, France. D.G. was the recipient of a PhD ANRS scholarship. S.B. was successively the recipient of a SIDACTION postdoctoral grant and an ANRS postdoctoral grant. V.M. was the recipient of an ANRS postdoctoral grant. We would like to thank Dr Cécile Butor for samples from healthy macaque, Drs Rafick-Pierre Sékaly, Jean-François Bureau, Fernando Arentzana-Seisdedos, Antonio Freitas and Ali Amara for helpful discussion and a critical reading of the manuscript and Richard Keatinge for his valuable help in preparing the manuscript.

Conflict of Interest Disclosure: M.M. is the CEO and Founder of Cytheris, B.A. and S.B. are employees of Cytheris. Cytheris develops recombinant interleukin-7 as a global immune enhancer and partly supported this work.

Authorship

S.B., S.R., D.G., R.P., V.M. and C.S. performed research; B.A., I.R. and M.M. contributed vital new reagents; P.L. participated to animal care; R.C and M.M. designed research and R.C. and S.B. analyzed data and wrote the manuscript.

REFERENCES

FIGURE LEGENDS

Figure 1. Injection of R-sIL-7gly rapidly induces major circulating T-cell loss in healthy Rhesus macaques.

Total lymphocyte (A), CD4+ T-cell (B), CD8+ T-cell (C) and CD20+ B-cell counts (D) were measured over 1 to 7 Days in 5 R-sIL-7gly-injected healthy Rhesus macaques (#14424: grey squares, #14694: grey circles, #26007-2 and #41127-2, hatched triangles and #40885-2: black triangles; left panels), in 2 untreated control animals (#21045 and #26007, white circles and white squares, right panels) and in an animal previously immunized against simian IL-7 and presenting high levels of neutralizing antibodies that also received R-sIL-7 at Day 0 (#17021, black diamonds, right panels). Three monkeys were sacrificed at Day 1 (#26007-2 and #41127-2) or at Day 7 (#40885-2). As compared to baseline, lymphocyte counts drop reached statistical significance at H24 (p<0.05) while both CD4+ and CD8+ counts were significantly reduced at H6, H12 (p<0.01), H24 and H48 (p<0.05). (E) Evolution of naive, central memory and effector memory CD4+ and CD8+ T-cell counts in R-sIL-7gly injected Rhesus macaques (#14424 and #14694, grey squares and circles) and untreated controls (#21045 and #26007, white circles and squares).

Figure 2. Evolution of chemokine receptor expression in R-sIL-7 treated macaques

A) CXCR4, CCR7, CCR6 and CCR9 expression were FACS-quantified on circulating CD4+ and CD8+ T-cells at H0, H6 and Day 2 following R-sIL-7gly injection in healthy Rhesus macaques (#14424, white symbols; #14694, black symbols). Representative FACS are shown for CD4 T-cells (left histograms) and CD8 T-cells (right histograms). Grey histograms represent chemokine receptor negative cells. Percentages of chemokine receptor positive cells are shown for each sample. B) The evolution of CXCR4, CCR6, CCR7 and CCR9 expression on naive, TCM and TEM CD4+ (left panels on each graph) and CD8+ (right panels) was
measured by FACS analysis in R-sIL-7gly injected healthy Rhesus macaques (#14424, white symbols; #14694, black symbols). For each T-cell subset, chemokine expression was measured at H0, H6 and D2 (left to right).

**Figure 3. R-sIL-7gly injection induces chemokine production in organs.**

CCL19, CCL20, CCL21 and CCL25 mRNAs were quantified in lymph nodes, jejunum, ileum, colon, rectum, lung and skin from healthy Rhesus macaques sacrificed at H0, H24 and D7 following R-sIL-7gly injection. Each symbol represents an individual quantification; grey and white symbols at H24 represent 2 individual macaques. Chemokine mRNA quantifications were normalized to HPRT mRNA in each sample and are presented as fold expression over a reference organ in which the expression of the given chemokine was low and stable over the experiment (i.e. jejunum for CCL19, skin for CCL20 and lung for both CCL21 and CCL25). Statistical analyses (Mann-Whitney test) between H0 and either H24 or D7 are shown on top. Horizontal bars represent medians.

**Figure 4. Quantification of plasma cytokines following R-sIL-7gly injection.**

Plasma concentration of various cytokines (IL-1β, IL-1RA, IL-5, IL-10, IL-12, IL-13, IL-16, MCP-1, Eotaxin, GCSF, IFNα, IFNγ, CCL3, CCL4, CCL5, CCL7, CCL27, CXCL8, CXCL9, MIF-3, NGFβ, SDF-1, SCF, TRAIL and SCGFβ) was quantified over a week in animals receiving a single dose of R-sIL-7gly injection (#14424 and #14694, black and white squares respectively) and in control animals using BioPlex™ assays. Control animals were #21045 and #26007 not receiving IL-7gly (black and white circles respectively) and #17021 immunized against R-sIL-7 before R-sIL-7gly treatment (grey diamonds). This animal presented high levels of anti-IL-7 neutralizing antibodies. Only cytokines demonstrating
significant variation are shown. A) Cytokines implicated in LN homing, B) cytokines implicated in migration into non-lymphoid organs, C) molecules playing a role in transendothelial migration.

Figure 5. R-sIL-7gly injection induces T-cell homing into organs

Rhesus macaques injected with R-sIL-7gly (80µg/Kg) were sacrificed at D1 (left pictures) and compared to untreated animals (right pictures). CD3+ cells were identified by immuno-histochemistry in skin (A), jejunum (B), ileum (C), colon (D), rectum (E) and lymph nodes (F) samples. CD3+ T-cell quantifications, performed on 7 to 10 fields (0.09 mm²) randomly selected from 4 slides for each organ in macaques sacrificed at H0, H24 and D7 following R-sIL-7gly injection are shown on the right. Statistical differences between H0 and either H24 or D7 are shown on top (Mann-Whitney test).

Figure 6. IL-7-induced Ki-67 and Bcl-2 expression in circulating blood and homing target organs

Initiation of cell cycling, as evidenced by the expression of Ki-67 (A) and cell survival capacity, as evidenced by Bcl-2 expression (B), were quantified in circulating CD4+ and CD8+ T lymphocytes in R-sIL-7gly injected Rhesus macaques (#14424 and #14694, black and white squares) as compared to non injected control animals (#21045 and #26007, black and white circles) over a 14 Days period following R-sIL-7gly injection. C) Ki-67+ cells were identified in lymph nodes, in Peyer’s patches (ileum), in the lamina popria (ileum, colon and rectum) and in the skin of animals sacrificed at H0, H24 and D7 following R-sIL-7gly injection. Ki-67+ T-cell quantifications, performed on 5 to 6 fields (0.09 mm²) randomly selected from 4 slides for each organ in macaques sacrificed at H0, H24 and D7 following R-
sIL-7gly injection are shown on the right. Statistical differences between H0 and either H24 or D7 are shown on top (Mann-Whitney test).
Figure 1

A
Lymphocyte counts (% from baseline)

RsIL-7 treated

Controls

B
CD4 T cell counts (% from baseline)

C
CD8 T cell counts (% from baseline)

D
CD20+ cell counts (% from baseline)

E
T cell counts (% from baseline)

Hours post treatment
Figure 2

A

CXCR4

CCR7

CCR6

CCR9

Positive cells in subset (%)

CD4

CD8

D0

D2

B

CXCR4

CCR7

CCR6

CCR9

Positive cells in subset (%)

CD4

CD8

N TCM TEM

N TCM TEM
Figure 3

L.N. CCL19 CCL21 CCL20 CCL25

Fold over reference

Days post R-sIL-7gly treatment
Figure 4

A

[Graphs showingCXCL12, MIF, CCL4, CCL3 levels over time post R-sIL7 gly]

B

[Graphs showing CXCL9, CXCL8 levels over time post R-sIL7 gly]

C

[Graphs showing IL-1beta, IL-1Ra levels over time post R-sIL7 gly]
Figure 5

A

Skin

B

Ileum

C

jejunum

D

Colon

E

Rectum

F

L.N.

Control (H0) vs. IL-7 (H24)

CD3+ T Cells/field

H0 H24 D7

0.001 0.006

0.003 0.01

N.S. N.S.

0.018

N.S.

0.027

0.05

N.S.
Figure 6

A

\[ \text{% of Ki-67+ cells} \]

CD4

CD8

Days post injection

B

\[ \text{Bcl-2 expression (MFI)} \]

CD4

CD8

Days post injection

C

<table>
<thead>
<tr>
<th></th>
<th>H0</th>
<th>H24</th>
<th>D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.N.</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Lymphoid follicles</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Ileum</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Colon</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Rectum</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Skin</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
Injection of glycosylated recombinant simian IL-7 provokes rapid and massive T-cell homing in rhesus macaques

Stephanie Beq, Sandra Rozlan, David Gautier, Raphaëlle Parker, Veronique Mersseman, Clementine Schilte, Brigitte Assouline, Iann Rance, Pascal Lavedan, Michel Morre and Remi Cheynier