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IL-21 promotes survival and maintains a naive phenotype in human CD4$^+$ T lymphocytes

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Abstract

IL-21 is a key T-cell growth factor (TCGF) involved in innate and adaptive immune response. It contributes to the proliferation of naive, but not memory T lymphocytes. However, the full spectrum of IL-21 activity on T cells remains unclear. Here, we demonstrate that IL-21 primarily maintains the expression of specific naive cell surface markers such as CD45RA, CD27, CD62L and CCR7 on human CD4$^+$ T lymphocytes and that the expression of CCR7 induces cell migration by means of CCL21 chemoattraction. These effects contrast with those of IL-2 which induced the marked proliferation of CD4$^+$ T lymphocytes, leading to an activated-memory phenotype. Nevertheless, IL-21 maintained cell cycle activation and expression of proliferation markers, including proliferating cell nuclear antigen and Ki-67, and triggered T-cell proliferation via TCR and co-stimulation pathways. Unlike IL-2, IL-21 decreased the expression of the anti-apoptotic Bcl-2 protein, which correlated with the absence of activation of the phosphatidylinositol 3′-kinase/Akt signaling pathway. Thus, IL-21 is a TCGF whose function is the preservation of a pool of CD4$^+$ T lymphocytes in a naive phenotype, with a low proliferation rate but with the persistence of cell cycling proteins and cell surface expression of CCR7. These findings strongly suggest that IL-21 plays a part in innate and adaptive immune response owing to homeostasis of T cells and their homing to secondary lymphoid organs.

Introduction

IL-21 is a member of the T-cell growth factor (TCGF) family, as are IL-2 and IL-15. TCGFs contribute to allograft rejection and the development of autoimmune and chronic inflammatory diseases by promoting activation, proliferation and infiltration of immune cells within the target organ (1–6). IL-21 may be involved in hematopoietic development and differentiation of NK cells, as well as in the activation and proliferation of B cells and co-stimulation of naive, but not memory, T cells (7–9). Unlike IL-2 and IL-15, IL-21 has no significant effect on the proliferation of T cells in the absence of anti-CD3 or other stimuli (7). Activated CD4$^+$ T cells, T_h2 cells for some authors (10) or memory CD4$^+$ T cells polarized toward T_h1 or T_h17 by others (11), also produce IL-21, suggesting that IL-21 may play an autocrine/paracrine role in the regulation of the immune response. IL-21 signals through the common γ chain as do IL-2 and IL-15, but it also binds with high affinity to a single receptor chain, IL-21 (7, 12). Ligation to their receptor subunits may contribute to the distinct effects of IL-2, IL-15 and IL-21 as ligand binding is associated with different intracellular signals (13), including Janus kinase (Jak) and signal transducers and activators of transcription (STAT) pathways. The phosphatidylinositol 3′-kinase (PI3K)/Akt pathway is also a common signaling component of cytokine members of the IL-2 family, as it is mediated by the S region of the intracellular tail of IL-2Rβ (14). In turn, the ability of IL-2 to activate Akt results in the inhibition of apoptosis and stimulation of cell proliferation as Akt clearly regulates the expression of Bcl-2 and c-myc (14). Akt activation may determine whether activated T cells undergo apoptosis or persist as regulatory/memory cells after robust immune activation, the key to peripheral T cell homeostasis. IL-21 is also a γc-dependent cytokine, but its receptor does not possess the IL-2Rβ chain as do IL-2R or IL-15R. Therefore, IL-21 may not induce proliferation of T cells via the same intracellular signaling pathway.

IL-21 is produced by activated CD4$^+$ T cells, but its functional effects on human naive CD4$^+$ T cells are poorly characterized and they seem to be less marked than its effects on CD8$^+$ T cells. Moreover, according to an increasing number of reports, the effects of IL-21 on human immune cells as

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compared with murine cells are conflicting (15–18). The present study pinpoints a novel role for IL-21 on naive T lymphocytes. This TCGF is necessary to conserve the naive phenotype of a pool of $CD4^+$ T lymphocytes which, however, does not prevent them from responding rapidly to potent stimuli. These data therefore suggest that one of the crucial functions of IL-21 is its implication in the survival and homeostatic proliferation of human naive $CD4^+$ T lymphocytes.

Methods

Reagents and cytokines

RPMI 1640 medium, FCS and β-mercaptoethanol (β-ME) were purchased from Sigma Chemicals (St Louis, MO, USA). PBS, penicillin–streptomycin, L-glutamine, MEM non-essential amino acids and sodium pyruvate were obtained from Gibco and Invitrogen (San Diego, CA, USA). Human AB serum was provided by the Blood Bank of the University Hospital of Geneva (Geneva, Switzerland). Ficoll-Paque™ Plus was from Amersham Biosciences (Uppsala, Sweden). Recombinant human IL-2 (rhIL-2) was obtained from Biogen, Inc. (Cambridge, MA, USA) and recombinant human IL-21 (rhIL-21) was a gift from D. C. Foster, Zymogenetics (Seattle, WA, USA).

Isolation of human CD4+ T lymphocytes and cultures

PBMCs were isolated from normal young donors by density-gradient centrifugation. T cells were separated from PBMC by negative selection with Dynabeads PanMouse IgG (Dynal Biotech, Oslo, Norway) and a cocktail of antibodies to CD16, CD19, CD14 and CD8 for selection of $CD4^+$ T cells (Dako, Copenhagen, Denmark). After negative selection, cells were washed with PBS and T cells stained with PE-conjugated mAb to CD45RO and FITC-conjugated mAb to CD4 (Dako). $CD45RO^-$ (naive) and $CD45RO^+$ (memory) $CD4^+$ T cells were subsequently separated on a FACSvantage® sorter (BD PharMingen™, San Diego, CA, USA). The selected $CD4^+$ cells were cultured for up to 3 weeks in RPMI 1640 medium supplemented with 10% heat-inactivated (HI) FCS, 5% HI AB serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine, 1% MEM non-essential amino acids and 0.1 mM sodium pyruvate, 5 mM β-ME (at 5×10^{-5} M) (referred to as medium), autologous feeder cells irradiated at 3500 rad and rIL-2 (25 ng ml^{-1}) or rIL-21 (25 ng ml^{-1}) in 5% CO_2 air humidified atmosphere at 37°C.

Cytokine determination

Samples of conditioned media were subjected to ELISA for the determination of IFN-γ, IL-4 and IL-10 as described previously (19). The sensitivity of all protein assays was 10–30 pg ml^{-1}.

T-cell proliferation assay in vitro

$CD4^+$ T lymphocytes were isolated as above and cultured for 7 days with rIL-2 (25 ng ml^{-1}) or rIL-21 (25 ng ml^{-1}). Cells were plated at 25×10^5 cells per well in U-bottom 96-well plates in enriched RPMI 1640 medium (as above) with medium alone, coated α-CD3 (1 μg ml^{-1}) or anti-human CD3 plus anti-human CD28-coated beads (Dynabeads® CD3/CD28 T cell expander kit, Dynal®). Cells were incubated in plates at 37°C for 72 h, followed by the addition of 1 μCi of [^{3}H]thymidine ([^{3}H]TdR) (Perkin Elmer Life Sciences, Inc.) for an additional 12 h. Cells were then harvested, [^{3}H]TdR incorporation per well was measured and the means assessed for each triplicate.

Cell staining for flow cytometry

The following mouse anti-human mAbs and isotype-matched control mAbs were purchased from BD PharMingen™: Cy-chrome-conjugated anti-CD4, PE-conjugated anti-CD45RO, anti-CCR7, APC-conjugated anti-CD62L, FITC-conjugated anti-annexin V, PE-conjugated propidium iodide (PI), FITC-conjugated Bcl-2, PE-conjugated proliferating cell nuclear antigen (PCNA), FITC-conjugated Ki-67 and isotype controls. The following mouse anti-human mAbs and isotype-matched control mAbs were obtained from Dako: FITC-conjugated anti-CD4, PE-conjugated anti-CD4, Cy-chrome-conjugated anti-CD45RA, FITC-conjugated goat anti-mouse IgG for anti-human CCR7 and irrelevant, isotype-matched controls. Murine anti-human mAb—FITC-conjugated anti-CCR7—was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Three- to four-color immunofluorescence was performed to assess surface marker expression on T cells activated by IL-2 or IL-21. After 1–3 weeks of culture, $CD4^+$ T lymphocytes were washed twice with PBS (completed with 2% serum AB and 1‰ NaN_3) and treated successively with FITC-, Cy-Chrome- and/or PE-conjugated mAbs on ice for 30 min and washed with PBS (completed with 2% serum AB and 1‰ NaN_3).

For analysis of DNA incorporation, $CD4^+$ T lymphocytes were cultured with rIL-2 (25 ng ml^{-1}) and/or rIL-21 (25 ng ml^{-1}) for 7 days. 10 μM of 5-bromo-2-deoxyuridine (BrdU) solution was added to culture medium at designated time points and cells were further processed for immunofluorescent staining as described in the instruction manual provided (BrdU flow kits, BD Pharmingen™).

For analysis of apoptotic cell death, $CD4^+$ T lymphocytes were cultured for 7 days in medium alone, with rIL-2 (25 ng ml^{-1}) or rIL-21 (25 ng ml^{-1}). Cells were harvested and stained with FITC-conjugated annexin V and PE-conjugated PI in accordance with the supplier's instructions (BD PharMingen™). For intracellular staining, $CD4^+$ T cells were isolated and washed once with PBS (completed with 2% serum AB and 1% NaN_3), and the intracellular proteins were determined according to the supplier's instructions using the Cytofix/Cytoperm Kit from BD PharMingen™. Cell staining was analyzed using FACSCalibur and CellQuest Software (BD PharMingen™).

Migration assay

Cell migration was evaluated using a chemotaxis microchamber technique as described previously (20). The chemoattractant recombinant human CCL21, anti-human CCL21 (both obtained from R&D Systems) or control medium was added at indicated concentrations to the 24-well tissue culture plates. A polyvinylpyrrolidone-free polycarbonate membrane with 8-μm pores for T lymphocytes (Nunc A/S) was layered onto the wells. Five hundred microliter of cell suspension (1×10^6 ml^{-1} of T cells) was seeded into the upper chamber. The chamber was incubated in 5% CO_2 air

humidified atmosphere at 37°C for 60 min. At the end of the incubation, filters were removed, and the cells that had transmigrated into the lower chamber were counted.

Fluorochrome 5-carboxyfluorescein diacetate succinimidyl ester labeling and analysis of T-cell proliferation in vitro

Human CD45RA$^+$ (naive) CD4$^+$ T cells were separated on a FACSvantage® sorter (BD Biosciences PharMingen, San Diego, CA, USA). The selected CD4$^+$ cells were labeled with fluorochrome 5-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probe, Inc., Portland, OR, USA), as described previously (21). CFSE was dissolved in dimethyl sulfoxide and added for 15 min to the cell suspension at a final concentration of 0.5 μM at 37°C. The reaction was stopped by the addition of PBS/10% FCS. The cells were washed in PBS/10% FCS, re-suspended into RPMI 1640 medium and cultured with IL-2 (25 ng ml^{-1}), IL-15 (25 ng ml^{-1}), IL-21 (25 ng ml^{-1}) or a combination of cytokines for 7 days. On day 7, cells were stained with Cy-chrome-conjugated anti-CD45RA and PE-conjugated anti-CD45RO (BD PharMingen™) for 30 min at 4°C. After staining, cells were washed once and re-suspended in 0.5 ml of PBS for analysis by flow cytometry on a Becton-Dickinson FACS Sort equipped with CellQuest Software. Live events were collected and analyzed by gating on to CD45RA$^+$ or CD45RO$^+$ CFSE$^+$ cells.

Calculation of the frequency of proliferating T cell

Proliferation of CD45RA$^+$ and CD45RO$^+$ T cells in response to cytokine stimulation was analyzed as described previously (22). With each round of cell division, the CFSE dye spread equally between the daughter cells. By means of the FACS acquisition software (CellQuest™), the total number of cells in each generation of proliferation was calculated and the number of precursors generating the daughter cells was determined by the following formula: $y/2^n$ (y = number of cells in each peak, n = number of cell divisions). The frequency of T-cell proliferation was then calculated by dividing the total number of precursors by the total number of CFSE-labeled cells.

Western blot analysis

CD45RA$^+$ T lymphocytes were purified as described above and cultured for 7 days with IL-2 (25 ng ml^{-1}). CD45RA$^+$ T lymphocytes were isolated and the cells were then starved overnight at 37°C in RPMI 1640 medium supplemented with 1% HI FCS. Cells were harvested and re-suspended at 4 × 10^6 cells ml^{-1} in RPMI medium supplemented with 1% HI FCS, and 500 μl was placed in 2-ml polypropylene tubes (Eppendorf) at 37°C. After 1 h, cells were stimulated with IL-2 (25 ng ml^{-1}) or IL-21 (25 ng ml^{-1}). After the indicated time of incubation, the reaction was stopped by the addition of 800 μl of ice-cold PBS and by centrifugation. Total cell lysate was prepared and analyzed by western blot (23). The blots were probed with anti-phospho Tyr^{701} STAT1, anti-STAT3, anti-phospho Tyr^{703} STAT3 and anti-phospho Ser^{473} Akt (Upstate Biotechnology). Secondary HRP-conjugated goat anti-rabbit antibodies were from Dako. Antibody-bound proteins were detected by the Uptilight hrp Blot Chemiluminescence substrate (Uptima).

Statistical analysis

Data were analyzed using the analysis of variance test, $*P < 0.05$ and $**P < 0.001$ being considered significant (Statview 5.1, SAS Institute Inc., Cary, NC, USA and GraphPad Prism 3.02).

Results

IL-21 preserves the naive phenotype of human T cells (CD45RA⁺)

To determine if IL-21 would induce proliferation and activation of human naive T lymphocytes, naive CD4$^+$ CD45RA$^+$ human T cells were purified, sorted and exposed to IL-21 and/or IL-2. Since IL-21 acts in synergy with IL-15 in enhancing CD8$^+$ proliferation and, in association with IL-2, modifies NK cell surface markers (24), we determined the role of IL-21 in naive CD4$^+$ CD45RA$^+$ human T cells alone or in the presence of IL-2. CD4$^+$ CD45RA$^+$ T lymphocytes incubated with IL-2 proliferated exponentially with time, as do T cells cultured with IL-2 plus IL-21, but to a lesser extent (Fig. 1A). As expected, IL-21 had little proliferative effect on CD4$^+$ CD45RA$^+$ T lymphocytes (7), and between days 14 and 21 of culture, the number of T cells decreased and they eventually died unless another TCGF was added. Cell sorting (day 0) revealed a typical CD45RA rather than CD45RO phenotype (Fig. 1B). Under all conditions, CD4$^+$ T cells presented a similar phenotype after 7 days of culture (Fig. 1B). However, when cells were cultured for as long as 14 and 21 days in the presence of rhIL-2, rhIL-21 or both simultaneously, their surface expression of CD45RA and CD45RO varied as shown in Fig. 1(B). In the presence of IL-2, human T lymphocytes tended to adopt a memory phenotype, with the CD45RO$^+$ marker replacing the CD45RA$^+$ surface marker (70% at day 7, 4% at day 14 and 3% at day 21 of CD45RA$^+$ CD45RO$^-$). With IL-21, 41% of T lymphocytes presented a naive phenotype at day 14, and a high percentage of CD45RA$^+$ cells was maintained until day 21 (70% of CD45RA$^+$ CD45RO$^-$) (Fig. 1B, Supplementary Table 1, available at *International Immunology* Online). T cells cultured in the presence of both IL-2 and IL-21 cytokines continued to present a naive phenotype at day 21 (27% of CD45RA$^+$ CD45RO$^-$), suggesting that IL-21 may modify the potent differentiating effect of IL-2.

If we consider the activated memory T-cell population to be the sum of the percentage in CD4$^+$ CD45RO$^+$ T lymphocytes and the percentage in double-positive cells in terms of activated CD4$^+$ CD45RA$^+$ T cells, as suggested by Arlettaz *et al.* (25), the percentage of T lymphocytes with a memory phenotype is even higher in cells treated with IL-2 than those treated with IL-2 + IL-21. Consequently, the T-cell population remaining at day 21 is enriched with CD4$^+$ CD45RA$^+$ T lymphocytes.

IL-21 maintains the co-expression of CCR7, CD45RA, and CD62L on CD4⁺ T lymphocytes

To further characterize the naive phenotype of CD4$^+$ T lymphocytes cultured with IL-21, several markers present on the cell surface of naive T cells were analyzed. IL-21 maintained a marked co-expression of CD45RA and CCR7 at

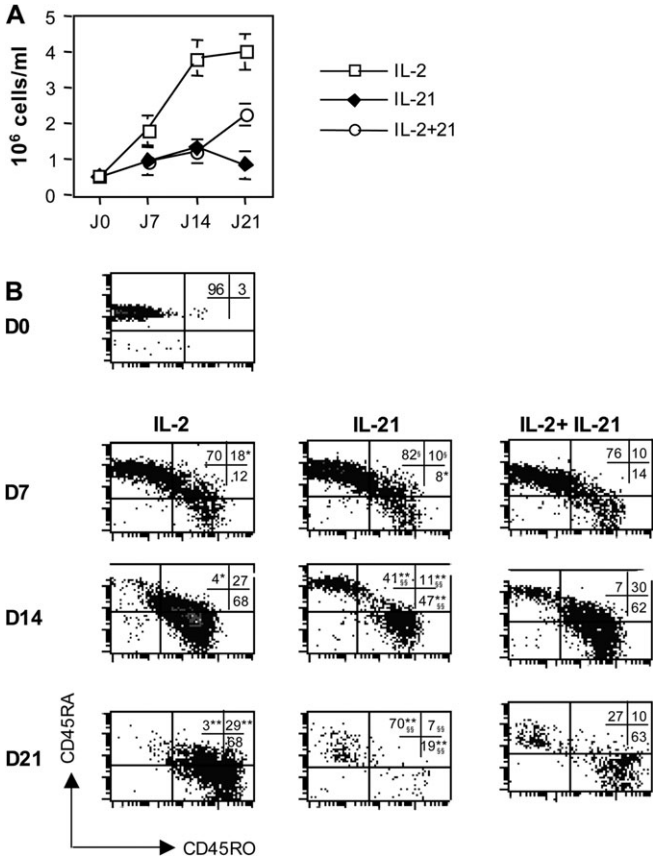

Fig. 1. IL-21 preserves the naive phenotype of T cells (CD45RA$^+$ T cells). Naive CD4$^+$ CD45RA$^+$ T cells were obtained from microbead-purified T cells by cell sorting. Purified naive T cells (96% of CD45RA$^+$ T cells as presented on the plot = day 0) were cultured for 7, 14 and 21 days with IL-2 (25 ng ml^{-1}), IL-21 (25 ng ml^{-1}) or both at the concentrations described in Methods. (A) Proliferation of CD4$^+$ CD45RA$^+$ T cells over time. The number of cells recovered at day 21 was as follows: IL-2 = 3.8 × 10^6 cells ml^{-1}, IL-21 = 0.81 × 10^6 cells ml^{-1}, IL-2 + IL-21 = 2 × 10^6 cells ml^{-1}. (B) The cells were analyzed for expression of CD45RA$^+$ versus CD45RO$^+$ on their cell surface. Cells were stained first with a Cy-chrome-labeled mAb to CD45RA$^+$ and then with PE-conjugated mAb to CD45RO$^+$. This experiment is representative of six individual experiments performed and data were analyzed using the analysis of variance test. Statistical analyses were performed to determine the differences between cells treated by IL-2 or IL-21 versus those treated by IL-2 + IL-21 (*P < 0.05 and **P < 0.001) and differences between cells treated by IL-21 versus IL-2 ($^{\S}P \leqslant 0.05$ and $^{\S\S}P \leqslant 0.001$) (Statview 5.1). The number of cells recovered at day 21 was as follows: IL-2 = 2 × 10^6 cells ml^{-1} (0.6 × 10^5 CD4$^+$ CD45RA$^+$ T cells ml^{-1}), IL-21 = 0.42 × 10^6 cells ml^{-1} (3 × 10^5 CD4$^+$ CD45RA$^+$ T cells ml^{-1}), IL-2 + IL-21 = 1.2 × 10^6 cells ml^{-1} (3.2 × 10^5 CD4$^+$ CD45RA$^+$ T cells ml^{-1}). Quadrants were generated based on staining profiles with control antibodies and counted events were gated on remaining living cells.

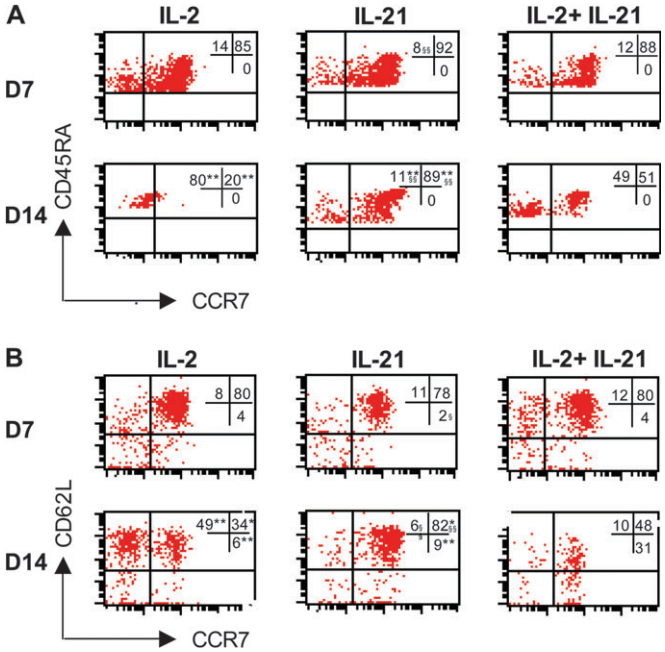

Fig. 2. Co-expression of CCR7, CD45RA and CD62L by CD4$^+$ T lymphocytes. Naive CD4$^+$ CD45RA$^+$ T cells were obtained by cell sorting from microbead-purified T cells. Purified naive T cells were cultured for 7 and 14 days with IL-2 (25 ng ml^{-1}), IL-21 (25 ng ml^{-1}) or both, and their cell surface was analyzed for the expression of CCR7, CD45RA and CD62L. The cells were stained with FITC-conjugated mAb to CCR7, Cy-chrome-conjugated mAb to CD45RA and APC-conjugated mAb to CD62L. (A) The cells were stained first with mAb to CCR7 and then with FITC-conjugated goat mAb to IgG and IgM and Cy-chrome-conjugated mAb to CD45RA. At day 14, the comparison of CD45RA$^+$/CCR7$^+$ T cells treated with IL-2 versus IL-21 yielded the following results: with IL-2 = 16 ± 2 versus IL-21 = 59 ± 15; P < 0.05. Quadrants were generated based on staining profiles with control antibodies. (B) The cells were stained first with FITC-conjugated mAb to CCR7, then incubated with APC-conjugated mAb to CD62L and finally stained with Cy-chrome-conjugated mAb to CD45RA. At day 14, the comparison of CD45RA$^+$/CCR7$^+$/CD62L$^+$ T cells treated with IL-2 versus IL-2 + IL-21 yielded the following results: with IL-2 = 25 ± 5 versus IL-2 + IL-21 = 47 ± 1.4: P < 0.05. Quadrants were generated based on staining profiles with control antibodies. Analysis was based on the expression of CD45RA/CCR7 or CD45RA/CCR7/CD62L at days 7 and 14. Data were analyzed using the analysis of variance test. Statistical analyses were performed to determine the differences between cells treated by IL-2 or IL-21 versus those treated by IL-2 + IL-21 (*P < 0.05 and **P < 0.001) and differences between IL-21 versus IL-2-treated cells ($^{\S}P \leqslant 0.05$ and $^{\S\S}P \leqslant 0.001$). Data were analyzed using the analysis of variance test, P < 0.05 and P < 0.0001 being considered significant (Statview 5.1).

their cell surface, i.e. 89% in IL-21-cultured T cells compared with 20% in IL-2-cultured T cells at day 14 (Fig. 2A), and despite the presence of IL-2, IL-21 maintained a higher expression of CCR7 on T cells (51% of CD45RA–CCR7 co-expression at day 14) (Fig. 2A, Supplementary Table 2, available at *International Immunology* Online). At the same time, we analyzed the co-expression of CD62L and CCR7 on CD45RA$^+$ CD4$^+$ T cells. As illustrated in Fig. 2(B), CD4$^+$ T cells in the presence of IL-21 expressed simultaneously high levels of both CCR7 and CD62L, whereas in the presence of IL-2, co-expression of these markers on the cell surface of CD45RA$^+$ CD4$^+$ T cells decreased with time. Co-culture of CD4$^+$ T cells with both IL-2 and IL-21 resulted in the expression of high levels of CCR7 and CD62L. Consequently, IL-21 is able to maintain CD4$^+$ T lymphocytes in a naive phenotype as demonstrated by the persistence of specific markers on their cell surface, such as CD45RA, CCR7 and CD62L. Not only did IL-21 have a marked direct effect on CD4$^+$ T cells but also diminished the differentiation and maturation of T cells induced by IL-2.

Chemotactic response of CD4⁺ T cells to recombinant human CCL21

CCR7 controls homing to secondary lymphoid organs, that in turn is crucial to homeostatic functions within the immune system. The interaction of CCR7 and its ligand SLC facilitates the entry of T cells into lymph nodes and Peyer's patches (26). In view of these reports and in order to further characterize the functional properties of the strongly enhanced expression of CCR7, we assessed the effect of IL-21 on chemotaxis. Human CD4⁺ T lymphocytes expressing a high percentage of CCR7 on their cell surface, i.e. cells cultured in the presence of IL-21, displayed a strong chemotactic response to recombinant human CCL21, but did not transmigrate in response to IL-8 (Fig. 3). The response to CCL21 was inhibited by a specific mAb to CCL21. These results confirm that naive CD4⁺ T cells expressing CCR7 on their cell surface respond to their ligand CCL21, which enables them to transmigrate in a double-chamber system assay.

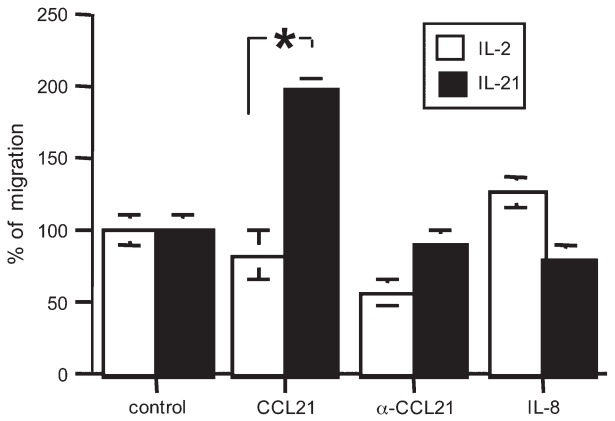

Fig. 3. Chemotactic response of CD4⁺ T cells to recombinant human CCL21. Naive T cells were cultured for 10 days with IL-2 (25 ng ml^{-1}) or IL-21 (25 ng ml^{-1}). Migration was assessed as described in Methods. Spontaneous migration was subtracted. Chemotaxis of human CD4⁺ T lymphocytes to recombinant human CCL21 was inhibited by a specific mAb to CCL21. Data represent the percentage of cells migrating to the lower chamber. Values are mean ± SEM of three separate experiments.

Differentiation and activation of CD4⁺ T cells by IL-21

The naive phenotype of T lymphocytes cultured with IL-21 did not indicate clearly if these cells survived, proliferated or underwent apoptosis. To determine the likelihood of CD4⁺ T lymphocytes differentiating in the presence of IL-21, cells (0.45×10^6 cells per ml) were cultured with IL-2 or IL-21. After 7 days of culture, the concentration of T cells treated with IL-2 amounted to 1.49×10^6 cells ml^{-1} and that of cells treated with IL-21 amounted to 0.83×10^6 cells ml^{-1}. All cells were stained with mAbs to CD45RA and CD45RO and sorted for a second time, gating on the CD45RA⁺ CD45RO⁻ cell population (Fig. 4A and B). The sorted cells were cultured once more at 0.2×10^6 cells per well with IL-2 or IL-21 for an additional 7 days at the same concentrations. As expected, the naive CD4⁺ T cells previously cultured with IL-2 presented an activated/memory phenotype when cultured once more with IL-2, 77% of cells expressing the CD45RO⁺ marker (6.8×10^5 CD4⁺ CD45RO⁺ T cells ml^{-1}), but only 18% of the T cells expressing CD45RA⁺ (1.6×10^5 CD4⁺ CD45RA⁺ T cells ml^{-1}) (Fig. 4B). In contrast, T lymphocytes pre-treated with IL-2 and then cultured with IL-21 maintained a naive phenotype, 66% of T cells expressing CD45RA⁺ (2.4×10^5 CD4⁺ CD45RA⁺ T cells ml^{-1}) versus 28% of T cells expressing the CD45RO⁺ marker (1×10^5 CD4⁺ CD45RO⁺ T cells ml^{-1}) (Fig. 4B). Simultaneously, naive CD4⁺ T cells previously cultured with IL-21 shifted into activated/memory phenotype when cultured afterward with IL-2, 35% of T cells expressing the CD45RO⁺ marker (1×10^5 CD4⁺ CD45RO⁺ T cells ml^{-1}) but only 16% of T lymphocytes expressing the CD45RO⁺ marker when cultured with IL-21 (0.3×10^5 CD4⁺ CD45RO⁺ T cells ml^{-1})

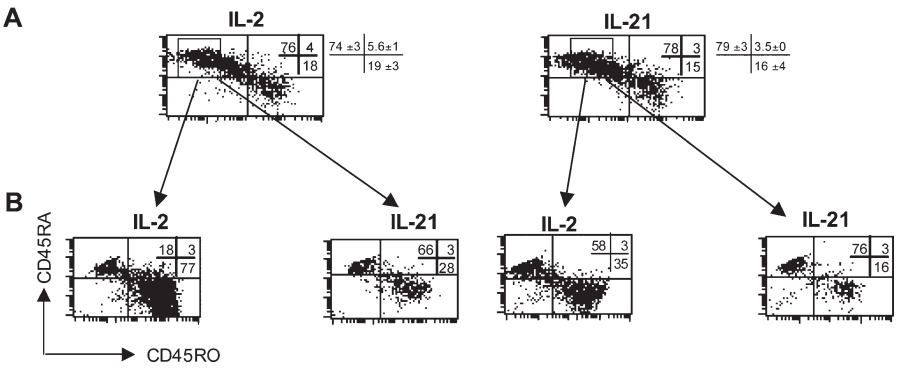

Fig. 4. CD4⁺ T cells cultured with IL-21 acquire an activated memory phenotype under stimulation. (A) Purified naive T cells were cultured for 7 days with IL-2 (25 ng ml^{-1}) or IL-21 (25 ng ml^{-1}). At day 7, the cells were stained first with Cy-chrome-labeled mAb to CD45RA⁺ and then with PE-conjugated mAb to CD45RO⁺. Cells were sorted a second time, gating on CD45RA⁺-CD45RO⁻ T lymphocytes (quadrant). (B) The sorted CD45RA⁺ T lymphocytes were cultured for an additional 7 days with IL-2 (25 ng ml^{-1}) or IL-21 (25 ng ml^{-1}), and their cell surface was analyzed for expression of CD45RA⁺ versus CD45RO⁺ as described above. After these 7 days of culture, cell concentrations under each condition were as follows: IL-2/IL-2 = 0.88×10^6 cells ml^{-1}, IL-2/IL-21 = 0.37×10^6 cells ml^{-1}, IL-21/IL-2 = 0.28×10^6 cells ml^{-1}, IL-21/IL-21 = 0.21×10^6 cells ml^{-1}. The FACS results are based on an analysis of all the cells, with equal events gated on living cells; hence, the variation in cell recovery does not skew the results or the percentage of CD45RA/CD45RO-positive cells. Values are mean ± SEM of three separate experiments and histograms are representative of three individual experiments performed.

(Fig. 4B, Supplementary Table 3, available at *International Immunology* Online). The latter observation confirms that T cells treated with IL-21 may be quiescent cells that maintain the ability to differentiate and proliferate in the presence of more potent TCGFs like IL-2.

To confirm that naive IL-21-treated CD4+ T lymphocytes can be reactivated, we assessed their proliferation and cytokine production. CD4+ T lymphocytes cultured for 7 days with IL-2 or IL-21 were placed in 96-well plates containing coated α-CD3 or anti-human CD3 together with anti-human CD28-coated beads and cultured for 72 h. The proliferation of the cells was determined by [3H]TdR incorporation. Under control conditions, the T cells pre-treated with IL-21 did not proliferate further, contrary to IL-2-pre-treated T cells which showed a slight persistent proliferation. IL-2-pre-treated T cells proliferated to a similar extent whether stimulated with α-CD3 or both anti-human CD3 and anti-human CD28-coated beads (Fig. 5A). In contrast, stimulation of IL-21-pre-treated T cells with α-CD3 induced very little proliferation contrasting with the massive proliferation observed with anti-human CD3 plus anti-human CD28-coated beads (Fig. 5A). Analysis of cytokine production in these culture conditions revealed that after stimulation with α-CD3 or anti-human CD3 plus anti-human CD28-coated beads, IL-2 and IL-21 up-regulated the production of IFN-γ, a T_h1-type cytokine (Fig. 5B). Of note, IL-21-treated T cells produced IFN-γ to a lesser extent when stimulated with α-CD3, whereas stimulation with both anti-human CD3 and anti-human CD28-coated beads prompted the marked production of IFN-γ; this effect was less pronounced on IL-2-treated T cells (Fig. 5B). These data indicate that although IL-21 plays a dual and dichotomous part on T cells by preserving a naive phenotype, it also permits their proliferative response to various stimuli (i.e. IL-2, α-CD3, co-stimulatory molecules) and induces the secretion of lymphokines.

IL-21 maintains cell division in naive CD4+ T cells

The above experiments suggest that CD4+ T cells exposed to IL-21 maintain their capacity to proliferate when exposed to potent stimuli. To further assess the influence of IL-21 on T-cell proliferation and viability, we labeled primary human CD4+ CD45RA+ and CD45RO+ T cells with CFSE or BrdU. In order to understand the mechanisms underlying the higher proliferation observed with IL-2 as compared with IL-21, CFSE-labeled CD4+ CD45RA+ T lymphocytes were cultured as described in the legend for 7 days (Fig. 6). On day 7, CFSE-stained cells were recovered and stained with Cychrome-conjugated anti-CD45RA (Fig. 6A) and PE-conjugated anti-CD45RO (Fig. 6B). Since CFSE spread equally between daughter cells after cell division, both pattern and frequency of proliferation of CD4+ T cells were analyzed by means of the CFSE-staining pattern, as described in Methods. In the presence of IL-2, 17% of CFSE-labeled CD4+ CD45RA+-gated T cells proliferated, contrasting with the massive proliferation of 88% in the CD4+ CD45RO+-gated T-cell population (Fig. 6A and B). With IL-21, only 12% of CD4+ CD45RA+-gated T cells divided, and CD4+ CD45RO+-gated T cells proliferated by ~4% (Fig. 6A and B). When cultured with IL-2 and IL-21, the frequency of proliferating CD4+ CD45RA+ and CD4+ CD45RO+ T cells was not increased compared with IL-2 treatment alone (Fig. 6A and B). To substantiate the above results, the proliferation of CD4+ T cells was also tested in the presence of IL-15, a potent inducer of T-cell proliferation, as well as IL-15 + IL-21. IL-15 induced the proliferation of both CD4+ CD45RA+ and CD4+ CD45RO+-gated T cells by 21 and 68%, respectively (Fig. 6A and B). We observed that both CD4+ CD45RA+ and CD4+ CD45RO+-gated T cells divided for multiple generations in the presence of both IL-15 and IL-21, with 29 and 91%, respectively, of CFSE-labeled T cells undergoing proliferation (Fig. 6A and B). This confirms that IL-21 significantly enhances the IL-15-driven proliferation of both naive and memory CD4+ T cells (11). To analyze cell cycle entry and progression of CD4+ T cells, sorted CD4+ CD45RA+ T cells were cultured for 7 days with IL-2, IL-21 or IL-2 plus IL-21 and labeled with BrdU at different time points. The incorporation of BrdU in newly synthesized DNA was analyzed by flow cytometry and by its staining associated with 7-AAD—a dye binding to total DNA—cells that had actively synthesized DNA were identified. As illustrated in Fig. 7, BrdU pulsing of actively proliferating CD4+ T cells was performed 3 h and 45 min before harvesting. CD4+ T cells cultured with

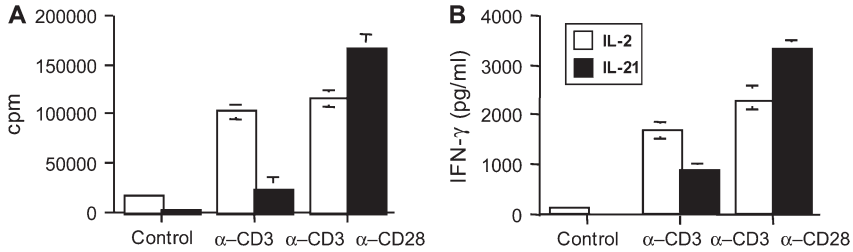

Fig. 5. Stimulation of CD4+ T cells cultured with IL-21. (A) Purified naive CD4+ T cells were cultured for 7 days with IL-2 (25 ng ml^{-1}) (open bars) or IL-21 (25 ng ml^{-1}) (closed bars). At day 7, the cells were washed once with PBS and cultured in plates coated with anti-human CD3 (1 μg ml^{-1}) or anti-human CD3 plus anti-human CD28-coated beads for 72 h and pulsed with [3H]TdR for the final 12 h. Radioactivity incorporated into proliferating cells was measured on a Betaplate™, Wallac, Perkin Elmer Life Sciences, Inc. (Boston, MA, USA). Values are means ± SEM from three separate experiments involving three different blood samples. (B) Purified naive CD4+ T cells were cultured for 7 days with IL-2 (25 ng ml^{-1}) (open bars) or IL-21 (25 ng ml^{-1}) (closed bars). At day 7, the cells were washed once with PBS and cultured in plates coated with anti-human CD3 (1 μg ml^{-1}) or anti-human CD3 plus anti-human CD28-coated beads for 48 h. Cultured supernatants were collected after 48 h and stored at −20°C until use. Cultured supernatants were tested by ELISA for content in secreted IFN-γ. Values are means ± SEMs from three separate experiments performed involving three different blood samples.

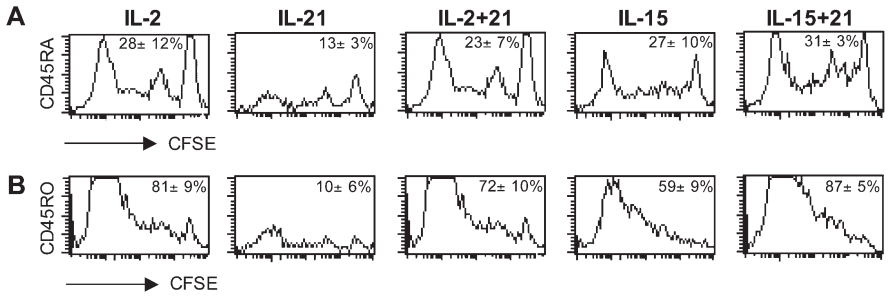

Fig. 6. IL-21 and IL-15 jointly increase the proliferation of CD45RA$^+$ T lymphocytes *in vitro*. Naive CD4$^+$ CD45RA$^+$ T cells were obtained from microbead-purified T cells by cell sorting. CFSE-labeled naive CD4$^+$ CD45RA$^+$ T cells were cultured for 7 days with IL-2 (25 ng ml^{-1}), IL-21 (25 ng ml^{-1}), IL-2 plus IL-21, IL-15 (25 ng ml^{-1}) or IL-15 plus IL-21. CFSE-stained cells were collected and stained with Cy-chrome-conjugated anti-CD45RA (A) and PE-conjugated anti-CD45RO (B). Data are represented as percent of proliferation of CFSE-labeled CD4$^+$ CD45RA$^+$ T cells (A) or CD4$^+$ CD45RO$^+$ T cells (B). The frequency of T-cell proliferation was determined as follows: the total number of cells in each generation of proliferation was calculated and the number of precursors that generated the daughter cells was determined by using the following formula: $y/2^n$ (y = number of cells in each peak, n = number of cell divisions) (23,24). The frequency of T-cell proliferation was then analyzed by dividing the total number of precursors by the total number of CFSE-labeled cells. Values are mean ± SEM of three separate experiments and histograms are representative of three individual experiments performed.

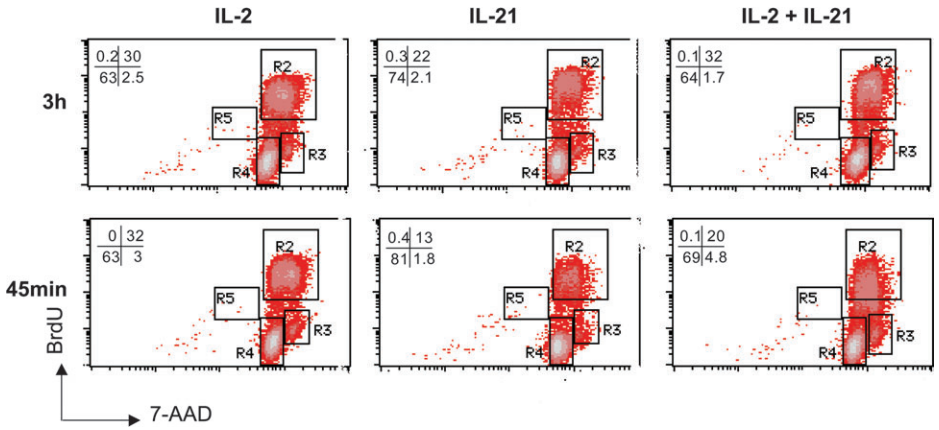

Fig. 7. IL-21 induces proliferation of CD4$^+$ CD45RA$^+$ T cells as assessed by active DNA synthesis. Purified naive CD4$^+$ CD45RA$^+$ T cells were cultured for 7 days with IL-2 (25 ng ml^{-1}), IL-21 (25 ng ml^{-1}) or both. At day 7, cells in culture were labeled with BrdU for the indicated time. Immunofluorescent staining of the cells was then performed as described in Methods with antibodies to CD45RA, BrdU and 7-AAD. Flow cytometric analysis was first gated on CD45RA$^+$ T cells and then region gates were applied to the 7-AAD versus BrdU dot plot as suggested in the BrdU Flow kit instruction manual. These region gates represent distinct cell cycle phases: R2 = S; R3 = G2 + M; R4 = G0/G1; R5 = apoptotic cells. For each marker, isotype controls were added. Data are representative of three similar and independent experiments.

IL-2, IL-21 or IL-2 plus IL-21 did not present major differences in their cell cycling subsets after 3 h of BrdU labeling (Fig. 7). However, after 45 min of BrdU labeling, we observed that in the presence of IL-21, CD4$^+$ T cells mainly resided in G0/G1 (R4), but few cells in phases S (R2) and G2 + M (R3) of the cell cycle. In the case of CD4$^+$ T cells cultured with IL-2, a higher number of cells were in the S (R2) and the G2 + M (R3) phases of the cell cycle, with a decreased number of cells in the G0/G1 (R4) phase, compared with cells exposed to IL-21. Similarly to IL-2 alone, the combination of IL-2 and IL-21 resulted in 69% of CD4$^+$ T cells in the G0/G1 (R4) phase, and a high number of cells in the S (R2) and G2 + M (R3) phases of the cell cycle. Consequently, the frequency of actively cycling cells is lower in CD4$^+$ T cells cultured with IL-21 than in those cultured with IL-2.

IL-21 regulates the growth and cell cycle progression of naive CD4⁺ T lymphocytes

Although IL-2 has the advantage of a stronger effect on proliferation than IL-21, it is also conducive to cell death via apoptosis. To compare the effects of both cytokines on apoptosis and cell cycle progression, we determined the expression of survival proteins and proliferating nuclear antigens. After 7 days of culture in control medium, >80% of CD4$^+$ T cells died according to double annexin V and PI staining, the signature of cell death (Fig. 8A). In contrast, in the presence of IL-2, cell viability was >80% (Fig. 8A), as confirmed by the absence of annexin V or PI staining. With the addition of IL-21, viability dropped to 44% but remained substantial in comparison with control medium. The moderate induction of apoptosis by IL-21 may correlate with the

down-regulation of the expression of Bcl-2, an anti-apoptotic member of the Bcl-2 family. After 7 days of culture in the presence of medium alone, IL-2 or IL-21, the expression of Bcl-2 protein in T cells was measured by flow cytometry. As shown in Fig. 8(B), $CD4^+$ T cells cultured in control medium did not express the Bcl-2 protein, indicating that all cells were apoptotic. In contrast, $CD4^+$ T cells cultured with IL-2 or IL-21 expressed high levels of the anti-apoptotic Bcl-2. It follows that even though markers of apoptosis may be slightly increased in naive $CD4^+$ T lymphocytes, IL-21 favorably affects naive $CD4^+$ T-cell viability. Finally, to confirm that IL-21 prompted cell cycle progression of purified naive $CD4^+$ T cells, the expression of proliferating nuclear antigens was determined. Markers of cellular DNA synthesis, PCNA and Ki-67—nuclear cell proliferation antigens expressed in all active stages of the cell cycle—were adopted. Progression of the cell cycle was monitored via the expression of PCNA and Ki-67 determined by flow cytometry. $CD4^+$ T cells cultured without any cytokines for 7 days failed to express any PCNA or Ki-67 whatsoever (Fig. 8C and D). IL-2, which effectively induced T-cell proliferation, increased the expression of the two proliferating nuclear antigens PCNA and Ki-67 by 88%. IL-21 also induced, although to a lesser extent, the up-regulation of both proliferating nuclear antigens with 67% of $CD4^+$ T cells expressing PCNA and 48% expressing Ki-67. A good correlation was found between the two markers PCNA and Ki-67. These findings provide evidence that IL-21 may control DNA synthesis, DNA repair and cell cycle. With regard to Figs 7 and 8, when similar experiments were performed at day 14, results were not different from those obtained at day 7 (data not shown).

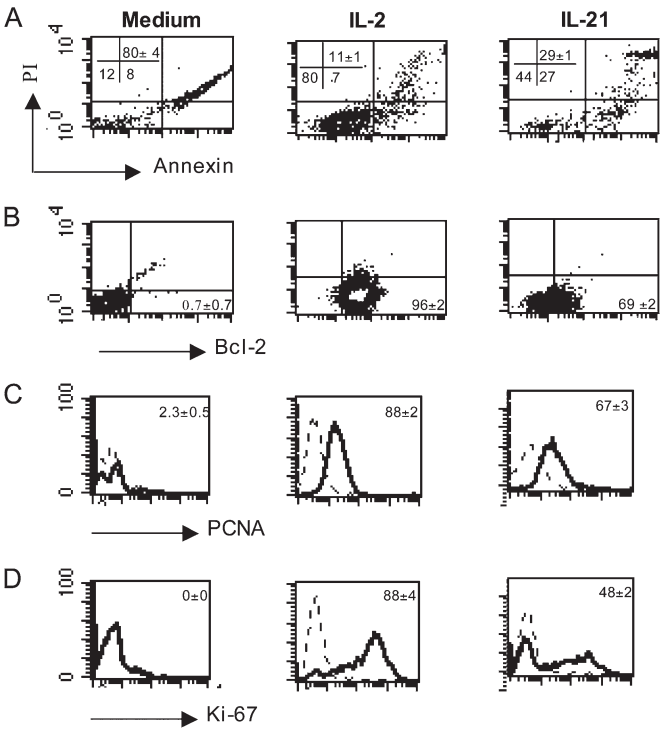

Fig. 8. IL-21 maintains the expression of survival and cell cycle proteins in naive $CD4^+$ $CD45RA^+$ T cells. Purified naive $CD4^+$ $CD45RA^+$ T cells were cultured for 7 days in medium alone, with IL-2 (25 ng ml^{-1}) or IL-21 (25 ng ml^{-1}). (A) At day 7, the cells were first stained with annexin V and PI. The percentages of double-negative subpopulations corresponding to viable cells are indicated in the quadrant. (B–D) The same cells were also tested for their intracytoplasmic levels of Bcl-2 (Fig. 7B), PCNA (Fig. 7C) or Ki-67 (Fig. 7D) by flow cytometry, as described in Methods. For each marker, isotype controls were added. Values are mean ± SEM of three separate experiments and dot blots or histograms are representative of three individual experiments performed.

IL-21 fails to signal through Akt

The weak effect of IL-21 on the proliferation of naive T lymphocytes may be due to differences in cell signaling. IL-2 mediated the activation of Jak1 and Jak3, which in turn induced the phosphorylation of STAT3, STAT5 and to some extent of STAT1. IL-2 also mediated T-cell proliferation through activation of the PI3K/Akt signaling pathway (27, 28). We therefore performed an experiment to compare signal transduction in human $CD4^+$ T lymphocytes activated by IL-2 or IL-21. A time course was run to determine phosphorylation of STAT1 and STAT3 under these stimuli (data not shown), and according to our results, maximum phosphorylation of STAT1 and STAT3 was reached after 7–15 min. As illustrated by Fig. 9, both IL-2 and IL-21 induced the phosphorylation of STAT1 and STAT3, but contrary to IL-2, IL-21 failed to induce the phosphorylation of Akt in T lymphocytes. These results may explain why IL-2 has a more potent effect on proliferation than IL-21.

Discussion

The population of activated T cells is continuously fed by a pool of naive CD4 and CD8 T cells, which process ensures they remain in the vicinity (29). Factors that contribute to the division of peripheral naive T cells are not all understood, but it is known that cytokines such as IL-7 play an important role in this process (30). It also has to be remembered that the number of peripheral blood naive T cells decreases proportionally with age. Our data demonstrate that in the human system IL-21 plays a crucial part in the survival of $CD4^+$ T lymphocytes with a naive phenotype. In mice, the effect of

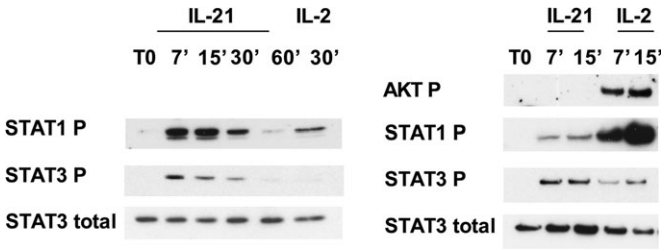

Fig. 9. IL-21 does not induce Akt phosphorylation in $CD4^+$ T cells. Isolated $CD45RA^+$ T cells (4×10^6 cells per 500 μl) were stimulated with IL-2 (25 ng ml^{-1}) or IL-21 (25 ng ml^{-1}) for the indicated time. Total cell lysates were analyzed by western blot, as described in Methods, with antibodies to Tyr701-phosphorylated STAT1 (PY-STAT1), Tyr703-phosphorylated STAT3 (PY-STAT3) and Ser473-phosphorylated Akt (PY-Akt). Equal loading was ascertained by anti-STAT3 mAb. Left and right panels represent two independent experiments performed at different time points, up to 60 min for the left panel. These results are representative of three independent experiments.

IL-21 on naive CD4+ T cells has not been extensively studied, but in *IL-21r*$^{-/-}$ mice the number of CD4+ T cells proved normal in both thymus and peripheral lymphoid organ (12). King *et al.* (5) demonstrated that IL-21 may play a major part in the development of diabetes in non-obese diabetic mice, due to an increase in T-cell proliferation and turnover coupled with the absence of survival signals. It thus appears that in mice IL-21 may favor the development of unstable, self-destructive T cells implicated in the pathogenesis of autoimmune disease (5). But clearly it needs to be emphasized that interspecies differences exist with respect to the biological effects of IL-21 (7).

We demonstrate that in the human system IL-21 promotes the survival and persistence of naive CD4+ T lymphocytes while maintaining the expression of CD45RA, CD62L and CCR7, the latter being related to the increased capacity of T cells to transmigrate in response to CCL21. IL-21 maintains a fraction of CD4+ T cells with a naive phenotype despite the addition of IL-2. Consequently, the effects of IL-21 do not duplicate those of IL-2, and the two cytokines have different but possibly complementary effects at various stages of T lymphocyte development. With TCR engagement (CD3) and co-stimulation (CD28), the activation and proliferation of naive CD4+ T cells pre-treated with IL-21 were as efficient as T cell priming by IL-2. The fact that in our experiments IL-21 failed to inhibit the production of IFN-γ conflicts with reports of IL-21 being conducive to T_h2-type T cells (10, 31), but it corroborates the work of others (8). The singular effect of IL-21 on human CD4+ T cells is corroborated by different reports (7, 10, 11, 18), as are its unique effects on other cell types, i.e. CD8+ T (32, 33), NK cells (7, 16, 31, 34) and B cells (35, 36). Others also suggest that IL-21, in synergy with other cytokines (IL-2, IL-15, IL-18), might fulfill specific functions on naive CD8+ T cells after induction of homeostatic proliferation (35), that it enhances CD8+ T-cell responses (24, 32) or the production of IFN-γ by human NK and T cells (8).

Control over T-cell survival and apoptosis is one of the keys to regulating peripheral immune homeostasis. Resorting to CFSE labeling (Fig. 6), we observed that IL-21 alone had no effect on CD4+ T-cell proliferation, but in association with IL-15, it enhanced the proliferation of CD45RA+ CD4+ T cells, similar to the synergistic effect observed on memory CD4+ T cells (11). By determining DNA synthesis by means of incorporated BrdU levels, we also demonstrate that even if the frequency is faintly decreased, CD45RA+ CD4+ T cells treated with IL-21 were able to synthesize DNA and to maintain cells in an active cycle. While IL-21 is known for its pro-apoptotic effect on B lymphocytes (37, 38), we demonstrate here that in CD4+ T cells, it increased the expression of Bcl-2, an anti-apoptotic molecule, similar to the effect of IL-2. These results, which confirm that IL-21 maintains the frequency of naive CD4+ T cells by inhibiting apoptosis, are also in keeping with the effect of a murine IL-21/Fc on growth and survival of murine T lymphocytes through the induction of Bcl-2 expression (39). Moreover, start and progression of the cell cycle are tightly regulated by the activation of cyclins and cyclin-dependent protein kinases, such as PCNA and Ki-67. Quiescent cells lack both, but after activation by cytokines (IL-2) or mitogens their expression is induced. The expression of these cell cycle markers in naive CD4+ T cells confirms that IL-21 helps to maintain the survival of naive CD4+ T cells. Finally, we demonstrate that the binding of IL-21 or IL-2 to their respective receptors triggers different pathways of intracellular signaling. The activation of Akt by IL-2 depends on PI3K-mediated signals provided by tyrosine residues within the IL-2Rβ chain (14), but the γc chain also engages signaling pathways through its tyrosine residues and may activate Akt, resulting in the up-regulation of Bcl-2 (27, 28). As the IL-21R complex lacks the IL-2Rβ chain, signaling through the γc chain may not be sufficient to induce full activation of the PI3K/Akt pathway in human CD4+ T cells, whereas in murine T lymphocytes, IL-21 induces survival through the activation of the PI3K signaling pathway (39). This is a further proof of IL-21 exerting differential effects on human and murine T lymphocytes.

In summary, IL-21 is able to maintain populations of naive CD4+ T cells and, by preventing the switch of one of the CD4+ T-cell fractions toward a memory phenotype, IL-21 emerges as a novel regulator of the immune response and as an additional cytokine involved in the transition of the immune response from innate to adaptive immunity. Our comparison of IL-21 and IL-2 has revealed some differences in the mechanisms of action underlying their regulation of T cells. These results may provide an additional step toward the understanding of IL-21 biology and novel insights into the potential implication of this cytokine in the pathogenesis of chronic autoimmune and inflammatory diseases.

Supplementary data

Supplementary Tables 1–3 are available at *International Immunology* Online.

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Abbreviations

BrdU	5-bromo-2-deoxyuridine
CFSE	fluorochrome 5-carboxyfluorescein diacetate succinimidyl ester
HI	heat-inactivated
Jak	Janus kinase
β-ME	β-mercaptoethanol
PCNA	proliferating cell nuclear antigen
PI	propidium iodide
PI3K	phosphatidylinositol 3′-kinase
rhIL-2	recombinant human IL-2
rhIL-21	recombinant human IL-21
STAT	signal transducers and activators of transcription
TCGF	T-cell growth factor
[^{3}H]TdR	[^{3}H]thymidine

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