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CXCL12 prolongs naive CD4 + T lymphocytes survival via activation of PKA, CREB and Bcl2 and BclXl up-regulation

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ABSTRACT

Background: Naive T lymphocytes recirculate through the body, traveling from secondary lymphoid organs through tissues and via lymphatic vessels and peripheral blood into other secondary lymphoid organs and into the bone marrow. In these tissues, lymphocytes are exposed to the chemokine CXCL12 which is abundantly produced in bone marrow and in lymph nodes by stromal cells. CXCL12 is known to drive lymphocytes chemotaxis and, in cells types such as stem cells, an antiapopototic effect has been described.
Methods: Here we analyzed the effect of CXCL12 exposure on naïve CD4 + T lymphocytes purified from peripheral blood by immunomagnetic negative isolation and cultured in a nutrient poor medium. We also studied, mainly by western blot analysis, the signaling pathways involved in CXCL12 action on naïve CD4 + T lymphocytes.
Results: We found that CXCL12-exposed cells survived longer than untreated ones and this prolonged lifespan was specific for resting naïve lymphocytes, while in vitro activated lymphoblasts died rapidly despite CXCL12 treatment. We demonstrated that the increased percentage of living cells observed upon CXCL12 administration was not due to induction of proliferation but to a prosurvival effect of this chemokine. Moreover, our data suggest that this prosurvival effect on naïve CD4 + T lymphocytes might likely be mediated by PKA-dependent CREB activation and consequent increased expression of the antiapoptotic factors Bcl2 and BclXl.
Conclusions: This newly reported activity of CXCL12 might contribute to the maintenance of the naïve T lymphocytes pool in vivo, which is needed to ensure a proper immune response to new antigens.

1. Introduction

Maintaining the naive T lymphocytes pool is essential to ensure an optimal immune response against novel antigens [1]. After intrathymic maturation, naive T cells colonize the periphery and recirculate through lymphoid organs until they encounter their specific antigen. Following antigen recognition, activation of naive T cells (characterized by phenotype changes and clonal expansion) occurs. This phase is then followed by contraction, so that the total number of peripheral cells remains in homeostatic equilibrium [2–6]. Lymphocytes that do not encounter their specific antigen continue to recirculate, keeping their naive phenotype. Despite thymic involution which begins early in the childhood, the proportion of circulating naive T cells remains relatively constant during adulthood [7]. This homeostasis is thought to be maintained by two main mechanisms: persistent thymic output of newly generated naive cells [8] and expansion of naive lymphocytes in the periphery [9]. Both these mechanisms have been confirmed by several studies included the detection of TCR excision circles (TRECs) in the peripheral blood of the elderly [10,11]. Along with these mechanisms, lifespan of resting naive T cells might also play a role in ensuring the maintenance of an untouched naive T lymphocytes pool.

CXCL12, also known as SDF-1α, is a widely studied chemokine, firstly described as growth factor for pre-B cells [12,13]. CXCL12 acts as a chemoattractant for T lymphocytes, monocytes and dendritic cells, and drives their homing to lymphoid organs [14]. It is abundantly produced in the bone marrow by stromal cells and CXCL12-abundant reticular cells (CAR) [15], and by stromal cells in lymph nodes [16]. Besides its function as a chemokine, CXCL12 has been demonstrated to have a co-stimulatory effect on T lymphocytes [17,18] and a cell type-dependent role in the apoptosis; in fact, it induces apoptosis of neurons [19] but has a pro-survival effect on stem cells [20] and granulosa cells in the

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preovulatory follicle [21]. CXCL12 binds on CXCR4, a highly conserved seven transmembrane G protein coupled receptor (GPRC) exclusive for CXCL12. The activation of CXCR4 by CXCL12 is mediated by a heterotrimeric G-protein, containing a $G_{\alpha i}$ and a $G_{\beta\gamma}$ subunit [22]. $G_{\alpha i}$ activation results in release of calcium via PLCβ as well as inhibition of adenyl cyclase (AC)-mediated cyclic adenosine monophosphate (cAMP) production [22]. CXCR4 stimulation induces calcium influx [23], the Ras–RAF–MEK–ERK [24] and the PI3K–AKT–NFkB [25] signaling cascades.

Alterations in the homeostasis of lymphocytes, in particular the decreased number of naive T lymphocytes and an inverted CD4/CD8 ratio are correlated with an increased incidence of infections [26] which have a relevant impact, among the others, on aged individuals and during rehabilitation. Therefore, unraveling the mechanisms that contribute to the maintenance of homeostasis could help to identify strategies to reduce the incidence of infections in medically complex patients. Here, we analyzed the survival rate of isolated naive CD45RA+ CD4+ T lymphocytes, exposed to CXCL12 and we observed, for the first time to our knowledge, that CXCL12 greatly prolongs the survival of CD4+ naive T lymphocytes cultured in a nutrient poor medium. This effect was specific for resting naive T cells and seems to be mediated by a PKA-dependent CREB activation and by an increased expression of the anti-apoptotic factors Bcl2 and BclXl.

2. Materials and methods

2.1. Lymphocytes isolation and cell culture

CD4+ CD45RA+ T lymphocytes were separated from peripheral blood mononuclear cells (PBMC). Leukocyte-enriched buffy coat from healthy donors were provided by the transfusion centre of Azienda Ospedaliera Universitaria Federico II. Written informed consent was obtained from each donor at the time of venous peripheral blood donation, in accordance with the Declaration of Helsinki, as approved by Azienda Ospedaliera Universitaria Federico II. All the experiments done by using blood donations were performed and analyzed anonymously, without any biographical reference to donors. Briefly, PBMC were obtained from buffy coats by centrifugation on a Ficoll-Hypaque plus (GE Healthcare, Milan, Italy) density gradient. PBMC were washed in PBS and CD4+ CD45RA+ T cells were isolated using the Naive CD4+ T Cell Isolation Kit II, (Miltenyi Biotech, Calderara di Reno, Italy), according to manufacturer's instruction. Activated lymphoblasts were obtained from naive T cells cultured for three days in complete medium (RPMI plus 10% FBS, 2 mM L-Glutamine, 1% Sodium Piruvate, 1% Non essential amino acids, 1% penicillin/streptomycin, all from ThermoFisher, Milan, Italy) in the presence of 2 μg/ml PHA (Sigma, Milan, Italy) and 20 U/ml IL-2 (Peprotech, Rocky Hill, NJ, USA). Cells were then cultured in RPMI (ThermoFisher, Milan, Italy) medium supplemented with 2% FBS (ThermoFisher, Milan, Italy) and 1% penicillin/streptomycin, with or without the adding of 100 ng/ml of SDF-1α/CXCL12 (Peprotech, Rocky Hill, NJ, USA). PD98059, H89 and 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) were purchased from Sigma-Aldrich (Milan, Italy). Cells were harvested at appropriated time points and analyzed by flow cytometry.

2.2. Flow cytometry analysis

Cells were stained with FITC conjugated anti CD45RA, PE conjugated anti CXCR4, PECy5 conjugated anti CD4 and APC conjugated anti CD3, all from Becton Dickinson (Milan, Italy). For propidium iodide (PI) staining, 2×10^5 cells were harvested at appropriate time points and 1 μg/ml PI (eBioscience, San Diego, CA) was added. Cells were acquired immediately after adding of PI. Acquisition and analysis were performed on a FACScalibur flow cytometer (Becton Dickinson) using CellQuest software.

2.3. Proliferation assay

Cell cultures were prepared in triplicate in microtiter plates. 100,000 cells were cultured for 72 h in the presence of anti CD3-coated magnetic beads (ThermoFisher, Milan, Italy), with or without 100 ng/ml soluble CXCL12. Anti CD3/CD28 coated magnetic beads were used as a positive control of proliferation. [^{3}H] Thymidine (Amersham International, Amersham, GB) was added to cultures during the last 6 h of incubation. Cells were then harvested (Tomtec) and radioactivity was measured with a betaplate scintillation counter (Wallac).

2.4. Western blot analysis

1×10^6 purified CD4 CD45 RA cells or activated lymphoblasts were incubated at 37 °C for different periods of time with CXCL12 at the concentrations indicated in figure legends, PMA/Ionomycin (50 ng/ml and 1 μg/ml respectively, both from Sigma Aldrich), or left alone. Lymphocytes were lysed in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml tosylphenylalanine chloromethyl ketone, 1 μg/ml leupeptin, 0.83 μg/ml chymostatin, 10 μg/ml soybean trypsin inhibitor, 1 mM sodium orthovanadate, and 0.5 mM dithiothreitol. The lysates were boiled for 5 min and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was performed using mAb to phospho-ERK1,2, ERK-1,2, phospho-CREB, Bcl2, BclXl, and β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA) and was developed using the SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher, Milan, Italy).

2.5. Statistical analysis

Statistical analysis were performed using Prism 5 software (GraphPad), using two-way Anova or Mann Whitney Test. All experiments were performed at least three times using cells from different donors.

3. Results

3.1. CXCL12 enhances naive T lymphocytes viability

To evaluate the ability of CXCL12 to induce long-lasting biological effects on naive T lymphocytes, we purified resting naïve CD4+ CD45RA+ lymphocytes from healthy donor's peripheral blood and we cultured them in a nutrient-poor medium in presence or absence of CXCL12. We evaluated naïve CD4+ CD45RA+ lymphocyte viability by flow cytometry every other day from day 1 to day 9 (Fig. 1A and B) by means of both propidium iodide (PI) staining (Fig. 1A) and changes in cell dimension and complexity (Fig. 1B). We observed that CXCL12-treated naïve CD4+ lymphocytes survived longer in a nutrient-poor medium compared to untreated cells. This effect was evident considering both the percentage of PI negative cells and cell morphology changes. In fact, at day 7, for example, only 20% of the untreated cells were PI negative (Fig. 1A; graph), compared to the 54% of CXCL12-treated cells (Fig. 1B; graph) ($p < 0.001$, $n = 6$), and the percentage of living cells based on morphological features was 52% for CXCL12-treated lymphocytes versus 27% for untreated controls ($p < 0.001$, $n = 9$). Notably, these data show the similarity of the results obtained by the two methods we used for cell survival detection. We then asked whether this effect was specific for naïve cells, or was common also to activated lymphocytes. To address this point we generated proliferating lymphoblasts by incubating naïve CD4+ T lymphocytes with PHA and IL-2 for 3 days. Lymphoblasts were then washed and cultured in 2% FBS medium, and their viability was evaluated every other day from day 1 to day 9 by analyzing cells morphology by flow cytometry (Fig.1C). To note, survival of activated blasts was decreased compared to naïve cells (Fig. 1B untr vs Fig. 1C untr), being lymphoblast survival percentage less than 60% by day 1, while CD4+ T lymphocytes survival percentage was 80% by the same day. More importantly, our experiments displayed that CXCL12 treatment did not increase the survival of proliferating lymphoblasts (Fig. 1C), this revealing the specific pro-survival action of CXCL12 on naïve T lymphocytes.

3.2. Resting naive lymphocytes and activated lymphoblasts express comparable levels of CXCR4

Next, we wanted to verify whether the specific action of CXCL12 only on naive T lymphocytes was merely due to different expression levels of the CXCL12 receptor CXCR4 on naïve T lymphocytes compared to proliferating lymphoblasts. In order to assess this issue, we quantified the expression of the CXCR4 membrane receptor by flow cytometry and we found that its expression levels were comparable in the two populations of cells (Fig. 2; 46,5% vs 48,6% in naïve cells and lymphoblasts, respectively). These data exclude that the absence of CXCL12-driven pro-survival effects on lymphoblast might be due to the absence of its receptor.

3.3. CXCL12 does not influence naïve T lymphocytes proliferation

The increased number of naïve CD4+ T lymphocytes detected upon CXCL12 treatment (Fig. 1) might also be due to a possible CXCL12-mediated activation of cells and induction of proliferation. Indeed, a

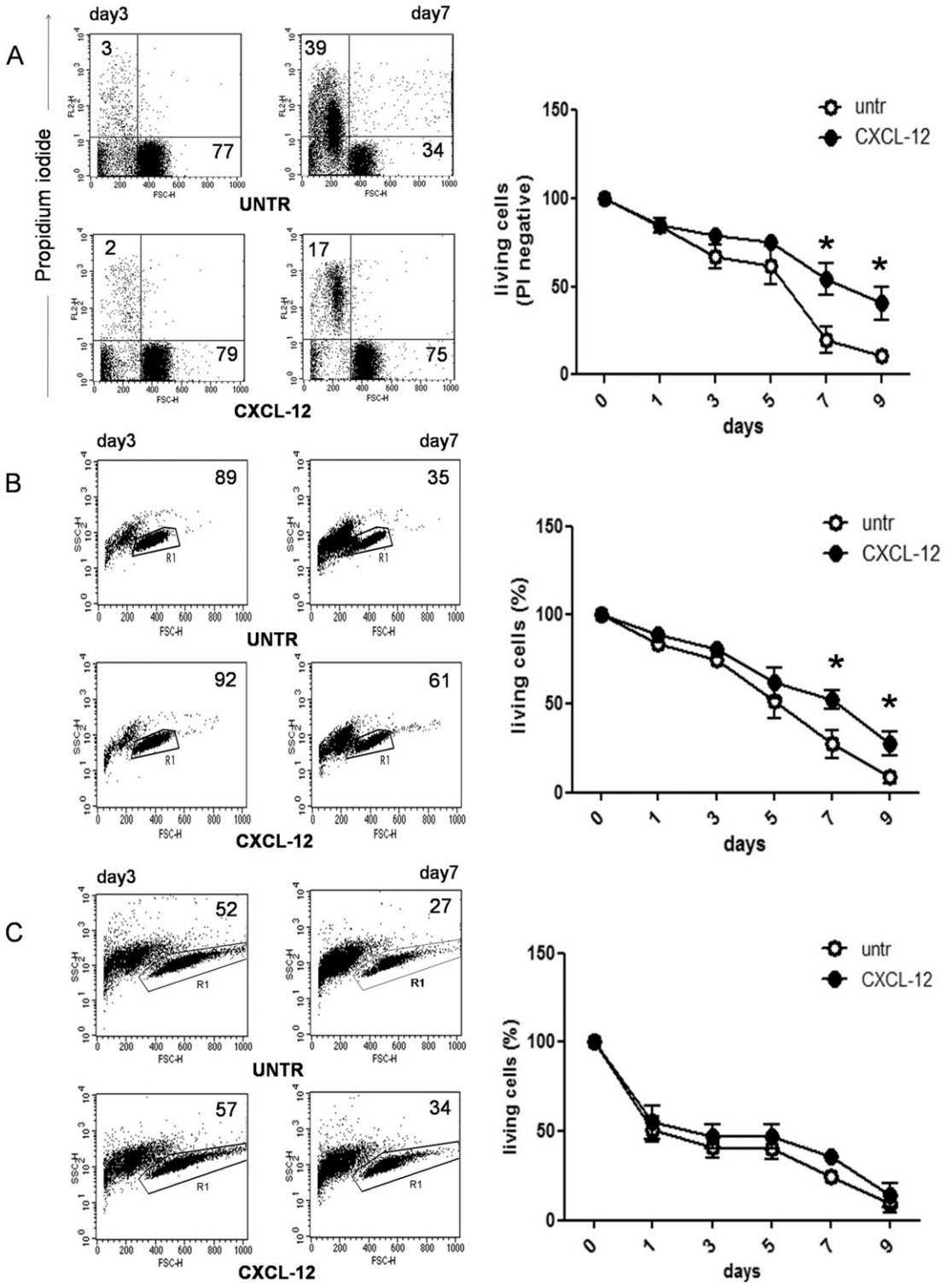

Fig. 1. CXCL12 promotes CD45RA + CD4 + T lymphocytes survival. A, Resting naive CD4 + T lymphocytes were separated from PBMC by negative selection, using immunomagnetic beads. Cells were cultured in a nutrient-poor medium (2% FBS) in the presence or absence of CXCL12 (100 ng/ml), and harvested every other day. Cells were harvested every other day, and stained with propidium iodide just before acquisition and analysis by flow cytometry. On the left, representative dot plots of propidium iodide (PI) staining versus cell size (x axis). Upper plots, untreated naïve cells at day 3 and day 7 of culture. Lower plots, cells treated with CXCL12. On the right, graph showing the percentage of PI negative cells over time. CXCL12 (black circle) increased the survival of naïve cells, compared to untreated lymphocytes (open circle) at day 7 and 9, ($p < 0.001$, $n = 6$). Asterisks indicates a statistically significative difference of $p < 0.001$. B, percentage of living cells was also calculated considering size and granulosity of cells by FACS analysis. Plots on the left represent a typical analysis of untreated (upper plots) and CXCL12 treated (lower plots) naïve cells at day 3 and day 7. CXCL12 increased cells survival at day 7 and 9, as compared to untreated controls ($p < 0.001$ $n = 9$, graph on the right). Asterisks indicates a statistically significative difference of $p < 0.001$. C, Activated lymphoblasts were obtained by stimulating for 3 days naïve CD4 + T cells with PHA (2 μg/ml) and IL-2 (20 U/ml). Cells were then washed and cultured in nutrient-poor medium for up to 9 days. Every other day cells were harvested and their viability was measured by flow cytometry analysis, considering size and granulosity. Plots on the left represent a typical analysis at day 3 and 7; graph on the right represent analysis of 6 independent experiments. CXCL12 (black circle) did not influence lymphoblasts survival ($p = 0.8$).

co-stimulatory effect of CXCL12 on unsorted CD4 + cells [17,18] had been previously reported. In order to establish the real role of CXCL12 on naïve CD4 + T lymphocytes survival, we analyzed their proliferation rate in response to anti-CD3 coated beads by measuring ^{3}H-thymidine incorporation in absence and presence of CXCL12. Our results showed that CXCL12 is not able to induce a co-stimulatory signal in naïve cells (Fig. 3). We therefore conclude that the increased number of living cells recorded upon CXCL12 treatment is not due to cellular activation and proliferation, but to a pro-survival effect.

3.4. The anti-apoptotic proteins Bcl2 and Bcl-Xl are up-regulated by CXCL12

To verify whether the pro-survival effect of CXCL12 on naïve cells could be due to the activation of anti-apoptotic pathways, we analyzed by Western Blot the expression of Bcl2 and BclXl, in CD4 + CD45RA + T lymphocytes treated or not with CXCL12 for 24 or 72 h. Interestingly, we found that CXCL12 strongly enhances the expression of Bcl2. Moreover, also the anti-apoptotic protein Bcl-Xl is up-regulated by CXCL12 (Fig. 4). These findings clearly show that CXCL12 increases the resistance of naïve CD4 + T lymphocytes to apoptotic death stimuli, which might likely be the mechanism through which CXCL12 improves survival in this cell population.

3.5. CXCL12 effect in naïve CD4 + T lymphocytes is PKA-dependent and mediated by the CREB activation

To better understand the mechanisms underlying the pro-survival effect of CXCL12, we analyzed some signaling pathways that might be associated to CXCR4 activation. In particular, since we have observed a Bcl2 over-expression in response to CXCL12 stimulation, and since a cAMP responsive element (CRE) sequence is present in the promoter region of Bcl2, we decided to analyze the phosporylation and activation of the cAMP responsive element binding protein (CREB). Interestingly, we found that CREB is over-phosphorylated in the presence of CXCL12 (Fig. 5A; 15 min and 60 min). This over-activation is rapid and physiologically transient as, at longer time points, the phosphorylation is no longer detectable both in untreated and treated cells (Fig. 5A; 18 h). PMA and Ionomycin (P/I) were used as control for optimal lymphocytes activation, as they activate PKC and open intracellular calcium storages. Consistently with our above observation of the absence of CXCL12 pro-survival effects on lymphoblasts (see Fig. 1C), we found that CXCL12 stimulation of lymphoblasts does not trigger CREB phosphorylation (Fig. S1). This results corroborate our hypothesis that CXCL12 pro-survival effects are mediated by CREB phosphorylation.

One of the principal activator of CREB is the cAMP-dependent protein kinase (PKA). To verify whether PKA activation is involved in the

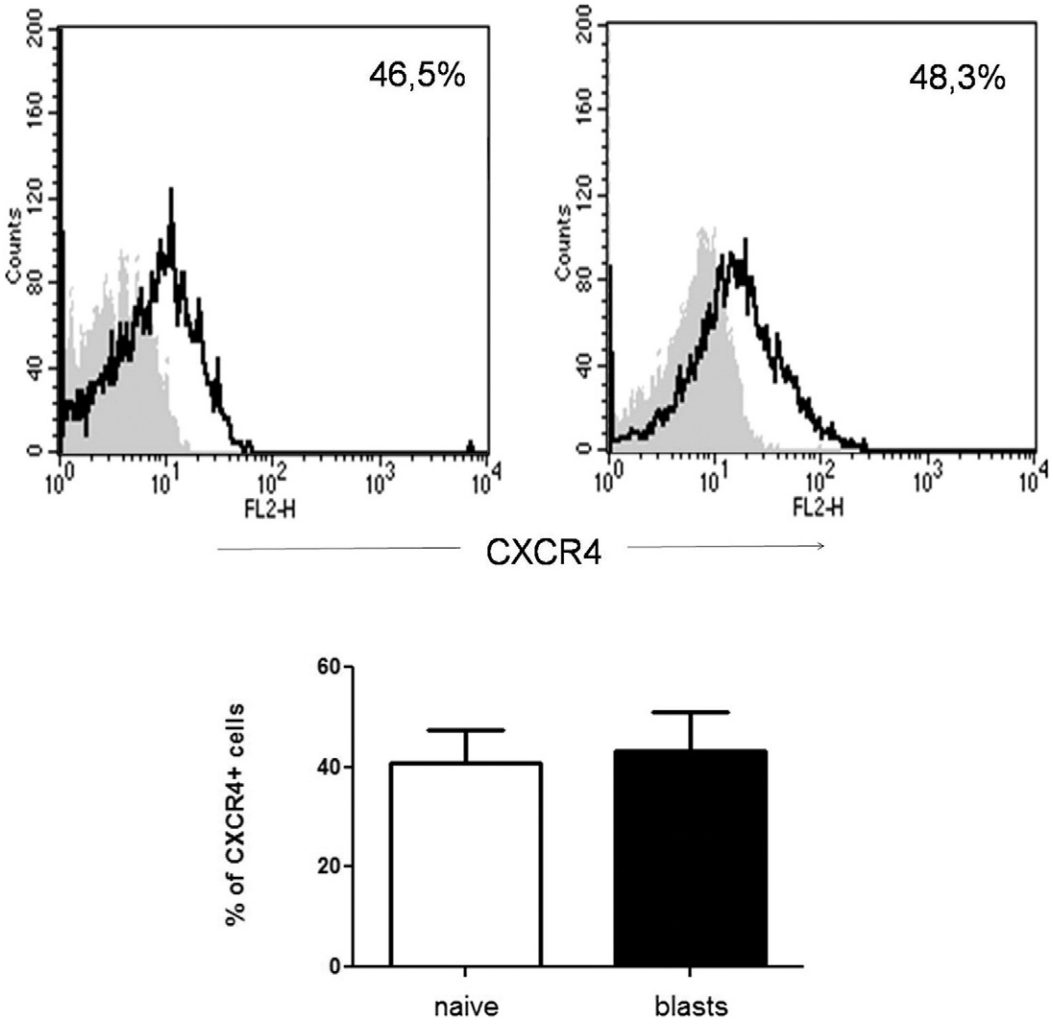

Fig. 2. Expression of CXCR4 on naive and activated CD4 + T lymphocytes. CXCR4 expression was evaluated by flow cytometry. Histogram plots show the expression of CXCR4 (open histogram) on naïve T cells (left plot) and on activated lymphoblasts (right plot). Gray histogram, isotype control. Numbers in plots indicate the percentage of CXCR4 expressing cells. Plots are representative of 6 different experiments. Bar graph shows the mean percentage of cells expressing CXCR4 ($n = 10$, $p = 0.8$).

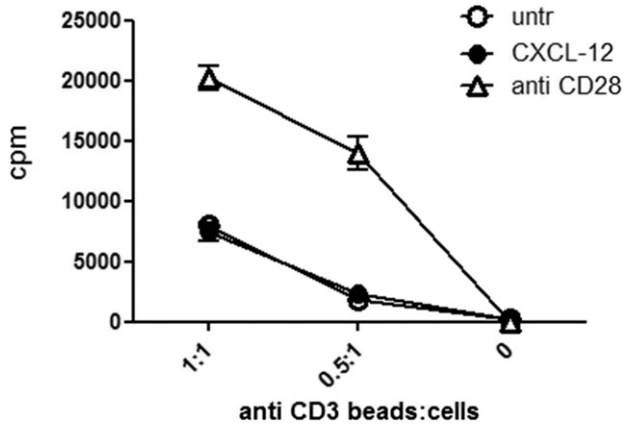

Fig. 3. CXCL12 does not influence proliferation of naive CD4+ CD45RA+ T lymphocytes. Naïve T cells were incubated for 72 h with anti-CD3 coated beads at different cells/beads ratios (x axis), in the presence (black circle) or absence (open circle) of CXCL12. As a positive control for costimulation, beads coated with anti CD28 were used (open triangle). In the last 16 h ^{3}H-thymidine was added and proliferation was evaluating by counting cpm using a plate reader beta-counter. Adding of CXCL12 to anti CD3 stimulated naïve cells did not increased their proliferative response compared to control (anti-CD3 coated beads only).

pro-survival effect of CXCL12, we cultured naïve cells in the presence of H89, a potent cell permeable inhibitor of PKA. We observed that PKA inhibition reduced cells viability of naïve CD4+ T lymphocytes in a nutrient-poor medium (Fig. 5B; H89 vs Fig. 1A; untr) thus indicating a role for PKA in naïve CD4+ T lymphocytes viability. Moreover, H89 reduced cell survival even in the presence of CXCL12 (Fig. 5B; H89 CXCL12); indeed, when H89 was added to the culture, the percentage of living cells decreased over time with a comparable kinetics in both CXCL12 treated and untreated cells, thus indicating that CXCL12 induced pro-survival effects are mediated by PKA activation (Fig. 5B). Furthermore, we observed that when 8-Br-cAMP, a cell-permeable cAMP analog that activates PKA, was added to naive cells, the percentage of living cells at day 5 and 7 increased and was comparable to that observed upon CXCL12 administration (Fig. 5C), confirming that PKA activation is involved in mediating CXCL12 induced naïve T lymphocytes survival.

Finally, we analyzed some members of the mitogen-activated protein kinase (MAPK) pathway, by evaluating ERK1/2 phosphorylation and we observed that treatment with CXCL12 did not induce ERK1/2 phosphorylation which results therefore not active in naïve cells (Fig. 5A). Consistent with this finding, the incubation of naive cells with the ERK1/2 inhibitor

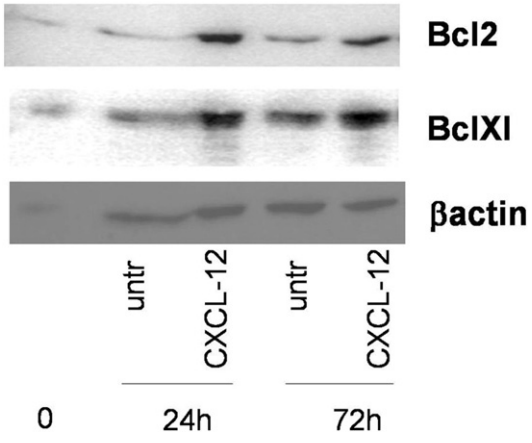

Fig. 4. CXCL12 induces the upregulation of Bcl2 and BclXl. CD4+ CD45RA+ resting T lymphocytes were incubated in the presence or absence of 100 ng/ml CXCL12 for the indicated times. Whole lysates were separated by SDS-PAGE and immunoblotted with the reported antibodies (BclXl, Bcl2). A typical immunoblot analysis is shown.

PD98059 did not influence CD4+ CD45+ T lymphocytes survival (Fig. S2). Viceversa, interestingly, CXCL12 rapidly induced ERK phosphorylation in activated lymphoblasts (Fig. S1).

4. Discussion

Here, we described a novel function of CXCL12 which we found able to influence naïve CD4+ T lymphocytes lifespan. Indeed, we showed that resting naïve T cells, cultured in a nutrient poor medium, display an increased survival when exposed to CXCL12. Naïve T lymphocytes die rapidly ex vivo in absence of stimuli, whereas the naïve T cell pool in vivo is maintained longer. One possible explanation for this difference is the homeostatic proliferation occurring in vivo, which is supported by the evidence of the reduction in TREC amount in naïve cells during aging [9,11]. However, our findings showing a prosurvival effect of CXCL12 on naïve T cells suggest that also exposure to CXCL12 and subsequent prolonged lifespan might contribute to maintenance of resting T cells. After maturation in the thymus, the majority of T cells continuously recirculate through the body, traveling through secondary lymphoid organs and bone marrow. CXCL12 is abundantly produced in bone marrow and by stromal cells in lymph nodes, where it attracts CXCR4+ B and T lymphocytes. Therefore, lymphocytes, circulating through bone marrow and lymph nodes, are constantly exposed to CXCL12. Our data clearly show that CXCL12 triggers prosurvival signals in naïve CD4+ T cells, therefore we hypothesize that this could be an additional mechanism by which naïve T cells compartment in vivo is kept. This hypothesis is consistent with a recent report of the role of CXCR4 in the homeostatic self-renewal of central memory CD8 T lymphocytes [27]. Another evidence supporting our hypothesis comes from the observation that moderate constant training improves immune status during aging increasing the number of circulating naïve cells [28,29] and that physical exercise can also upregulate the expression of CXCR4 [30].

Interestingly, our experiments showed that the prosurvival effect of CXCL12 was specific for resting naïve CD4 T cells, while we demonstrated that CXCL12 did not influence the lifespan of activated lymphoblasts. We reasoned that this difference might be due to a different amount of CXCL12 receptors expressed by the cells or to different signaling pathways activated downstream CXCR4 in the two cell types. Our experiments revealed that the intensity of expression of CXCR4 is comparable in the two populations of lymphocytes, whereas the early signaling pathways activated upon CXCL12 exposure change very much between them.

In the attempt to unravel the signaling triggered by CXCL12 and enhancing naïve CD4+ T lymphocytes survival, we found that the PKA substrate CREB is phosphorylated by CXCL12. This suggests that a PKA- and CREB-dependent mechanism might be responsible for the prosurvival effect of CXCL12 on naïve cells. This hypothesis is strengthened by the absence of CREB stimulation in lymphoblasts where the prosurvival effect is not occurring. On the same basis, the MAPK–ERK pathway does not seem to be involved in CXCL12-induced lifespan increase in naïve cells, as we didn't observe ERK 1,2 phosphorylation, which, by contrast, was rapidly induced by CXCL12 in lymphoblasts where survival is not enhanced. Moreover, the prosurvival effect of CXCL12 was completely abrogated by the presence of H89, a cell permeable PKA inhibitor, whereas survival increases when a stable cAMP analog is added to the culture. These findings strongly indicate PKA as a key player in mediating the survival signaling induced by CXCL12.

Since PKA is activated by cAMP which is produced by adenyl cyclase, our finding that, in naïve CD4+ T lymphocytes, CXCL12 induces PKA activation appears controversial with the knowledge that, according to the classical CXCR4 downstream signaling, AC should be inhibited by CXCL12 stimulation and consequent $G_{\alpha i}$ release. This apparent inconsistency could be explained by the evidence that in different cell lines the same GPRC could be associated alternatively to $G_{\alpha i}$ or $G_{\alpha s}$, inducing different downstream signals when activated [31]. Moreover, activation of

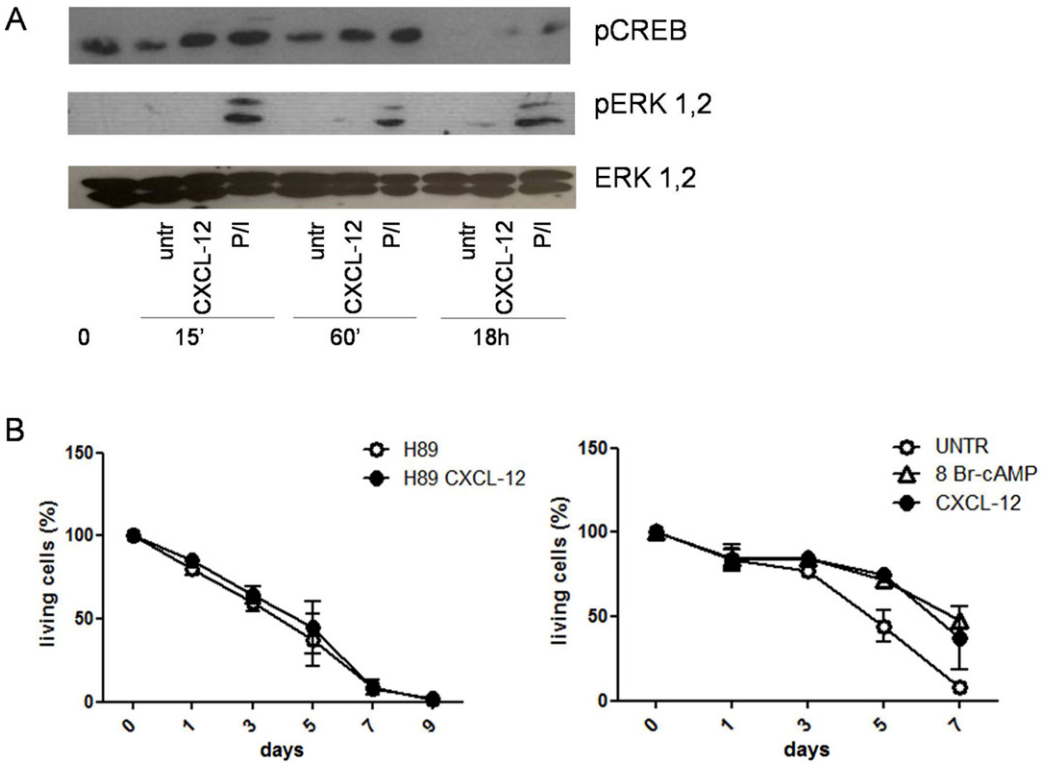

Fig. 5. CXCL12 induced survival effect is mediated by CREB activation. A, naive T lymphocytes were incubated for the indicated times with CXCL12 (1 μg/ml), PMA/Ionomycin or left untreated. The activation of CREB and ERK were analyzed by SDS-PAGE and western blot. Total ERK was used as normalizer. CXCL12 induced CREB phosphorylation in naive cells, while ERK was not activated by CXCL12. B, on the left, graph showing the viability of CD4+ CD45RA+ naive T lymphocytes incubated for up to 9 days with or without CXCL12 in nutrient poor medium, in which the protein kinase A (PKA) inhibitor H89 (10 μM) was added. Cells viability was measured by flow cytometry, considering size and granulosity of cells. The pro-survival effect of CXCL12 was abrogated by the presence of H89. B, on the right, the graph shows the viability of CD4+ CD45RA+ cells, cultured alone or in the presence of CXCL12 or of 8 Br-cAMP (100 μM). The prosurvival effect of CXCL12 was mimicked by the action of 8 Br-cAMP.

PKA subsequent to CXCR4 engagement has been previously reported in embryonic retinal ganglion cells [32], in monocytes [33] and in naïve CD4+ lymphocytes [34], supporting our results. In addition, although for years it has been believed that CXCL12 and CXCR4 had an exclusive relationship, recent evidence demonstrated that CXCL12 can also bind to the CXCR7 receptor [35,36]. CXCR7 activation might be taken in account in order to explain the unusual activation of PKA we found. This point deserves to be further investigated, although CXCR7 does not signal through a G protein, but rather via a β-arrestin [37] dependent signaling and this would not fit with our observation of a PKA-dependent induction of survival.

The different signaling pathways activated by CXCL12 on resting naïve lymphocytes and on activated lymphoblasts also explain why, in our experimental settings, CXCL12 did not have the costimulatory effect on proliferation reported by other groups who showed that CXCL12 increases the proliferative response of CD4+ T lymphocytes [18] being CXCR4, with CCR5, part of the immunological synapse. This difference is likely due to the fact that here we analyzed naïve resting CD4+ cells and not total circulating lymphocytes which include also activated and memory cells. The effects observed in a mixed population depend on the subpopulation present in the higher proportion and, in total lymphocytes, naïve T cells represent a minor population whose non-responsiveness to the costimulation exerted by CXCL12 could be masked by the ready response from other CD4 subsets.

Taken together, our results describe a long lasting prosurvival effect of CXCL12 on naïve T lymphocytes. This effect is mediated by early PKA and CREB activation, and is sustained by the induction of Bcl2 and BclXl. Further studies are needed to verify whether this mechanism could be exploited to improve immune response where necessary, such as, in complex patients care.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2016.09.007.

Contributions

LV performed experiments, acquired and analyzed data and drafted the manuscript; EF analyzed the data and revised the manuscript; SDS performed experiments; LG and AF performed experiments, acquired and analyzed data; LR designed the study, interpreted the data and revised the manuscript; GMR revised the manuscript. All authors approved the submitted version.

The authors report no relationships that could be construed as a conflict of interest.

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Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

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