

IL-7 improves the fitness of regulatory T cells for adoptive transfer

Paolo Monti¹, Ilaria Cosorich¹, Jessica Filoni¹, Carla Di Dedda¹, Arianna Ferrari¹, and Lorenzo Piemonti¹

¹IRCCS Ospedale San Raffaele

May 18, 2023

Abstract

Adoptive regulatory T cell (Treg) transfer represents a potential therapeutic option to control immune responses in organ transplantation, graft vs host disease, and autoimmunity, including type 1 diabetes. Treg for adoptive therapy are traditionally sorted and expanded *in vitro* with high doses of IL-2, showing stability and suppressive capacity, but with some limitations in terms of long-term survival once infused in patients. Here, we tested a novel expansion protocol in which we added IL-7 (IL-7 method, IL-7M) to the traditional standard method (StM) using IL-2. We showed that naïve Treg express significant levels of CD127 and robustly respond to IL-7 by phosphorylating STAT-5. Naive Treg expanded with the IL-7M were highly enriched in CD45RA⁺CD62L⁺CD95⁺ showing a reduction in the final cell yield and suppressive function. Treg expanded with the IL-7M preserved telomere length and were more resistant to cytokine withdrawal and fas-mediated apoptosis. Overall our data indicate that it is possible to expand naïve Treg in the presence of IL-7 to generate a final Treg product enriched in poorly differentiated CD45RA⁺ cells and with better resistance to stress and apoptosis, potentially improving the long-term survival of Treg in adoptive transfer protocols.

Introduction

CD4⁺CD25⁺FOXP3⁺regulatory T cells (Treg) are a T cell subset specialized in the regulation of the immune response and maintenance of immune tolerance (1)(2). These properties underscored their potential for adoptive immunotherapy to control transplant rejection (3), graft vs host disease (4), and autoimmunity (5), including type 1 diabetes (T1D) in more than 50 active or completed clinical trials (6). While polyclonal Treg are still the cells more used in clinical adoptive immunotherapy, CAR or TCR transgenic Treg are expected to become widely available in the near future (7). Adoptive immunotherapy with polyclonal Treg involves their isolation from peripheral blood and the use of *in vitro* expansion protocols to obtain a final Treg product of therapeutic value in terms of cell yield, stability and suppressive function. In patients with T1D, the “first-in-man” clinical trial with polyclonal Treg proved feasible and safe but showed some limitations in terms of long-term survival of adoptively transferred Treg (8). Specifically, it showed a bi-phasic exponential decay kinetic with a short-lived Treg subset (75-90%) with a half-life of few days, and a long-lived Treg subset (10-25%) detectable up to one year after infusion. Notably, while Treg after expansion displayed a CCR7⁺CD45RO⁺CD45RA⁻central-memory phenotype, Treg that survive longer in patients displayed a CCR7⁺CD45RA⁺CD45RO^{-/+}phenotype similar to that of less differentiated naïve or memory stem T cells. Treg are traditionally isolated in bulk as CD4⁺CD25^{high}CD127^{low} (9) and expanded using anti CD3/CD38 microbeads in combination with high doses IL-2 which act as a potent mitogen but also pro-differentiating cytokine leading to the generation of memory subsets (10). T cells proliferate also in response to homeostatic cytokines such as IL-7 and IL-15 that are permissive for expansion but preserves a poorly differentiated phenotype. Studies conducted on conventional T cells showed the capacity of IL-7 (7) or combination of IL-7 and IL-15 (11) to generate memory stem T cells from naïve precursors after expansion. Treg express low levels of the IL-7Rα (CD127) (9). However, we have previously showed the capacity of human naïve Treg to respond to and proliferate in the presence of IL-7 *in vitro* (12). Notably, in conditions of Treg depletion,

IL-7 contributes to the reconstitution of the Treg compartment also in vivo in patients treated with the anti CD25 monoclonal antibody basiliximab (13).

We hypothesized that modifications to the in vitro expansion protocol that are permissive for Treg expansion, while preserving a poorly differentiated CD45RA⁺ phenotype can be associated to an increased resistance of Treg to stress signals and therefore improve the long-term survival of Treg once infused in patients in adoptive transfer protocols. To test our hypothesis we sorted Treg (CD4⁺CD25^{high}CD127^{low}) with a naïve CD62L⁺CD45RA⁺CD95⁻ phenotype and added IL-7 to the culture medium during the expansion. Our findings suggest that expansion in the presence of IL-7 generated a Treg product with an immature phenotype and improved resistance to stress and apoptosis.

Results

Responsiveness to IL-7 in circulating naïve and memory Treg subsets

As for conventional T cells, also circulating Treg can be categorized according to surface phenotypes corresponding to naïve or memory subsets. According to the expression of CD45RA, CD62L and CD95 we determined the relative proportion (%: median, IQR) of Treg naïve (10.1, 7-16.3 Treg-n; CD45RA⁺CD62L⁺CD95⁻), Treg stem cells memory (1.2, 0.6-2.6 Treg-scm, CD45RA⁺CD62L⁺CD95⁺), Treg central-memory (44.8: 32.5-59.6 Treg-cm, CD45RA⁻CD62L⁺CD95⁺), Treg effector-memory (42.5, 35.8-52.1 Treg-em, CD45RA⁻CD62L⁻CD95⁺) and Treg effector-memory re-expressing CD45RA (0.4, 0.2-0.6 Treg-emra, CD45RA⁺CD62L⁻CD95⁺) in peripheral blood samples from 16 adult healthy subjects (Figure 1A and B). In parallel we determined the relative proportion of the same subsets in circulating conventional CD4⁺ T cells (Supplementary Figure 1A and 1B). Using this phenotypic classification we found all the subsets described for conventional T cells, including Treg with a memory stem cell phenotype that have never been described before. Since Treg are defined as CD127^{low} cells (14), we measured (MFI; median, IQR) the expression of CD127 in all the Treg subsets identified and compared it to the one of conventional bulk CD4⁺ T cells (3205, 2064-4692) and CD19⁺ B cells (57, 49-69) that do not express CD127 (15) (Figure 1C). Treg-n showed the highest expression of CD127 (480, 316-611) followed by Treg-scm (432; 304-515), Treg-cm (328; 246-390), Treg-em (173, 122-236), and Temra (132, 101-159). We next incubated Treg with IL-7 (10ng/ml for 1 minute) and measured the phosphorylation of STAT5 to determine whether CD127 was functional and sufficiently expressed to elicit intracellular signaling (Figure 1D). We compared STAT-5 phosphorylation (MFI; median, IQR) to the one of conventional CD4⁺ T cells (8056, 6730-10526) and CD127⁻ B cells (167, 126-168). Treg-n showed the strongest STAT-5 phosphorylation (3386, 2794-4629) followed by Treg-scm (2540, 1972-2817), Treg-cm (1500, 950-1827), Treg-em (779, 364-909), Treg-emra (576, 332-776). These data show that Treg were responsive to IL-7, especially those with a naïve phenotype or a stem cell memory phenotype, even though their response in terms of STAT-5 phosphorylation was far less intense than the one of conventional CD4⁺ T cells. Based on these observations, we tested the capacity of IL-7 to trigger Treg expansion in comparison or in combination with IL-2 (Figure 1E). Treg showed a limited fold expansion (median, IQR) in response to IL-7 (10, 8-11) while expanded vigorously in the presence of IL-2 (85, 68-95). The combination of IL-7 and IL-2 induced an intermediate level of Treg expansion (54, 34-61) that was unexpectedly less than the one with IL-2 alone. We interpreted these data as the result of competition between IL-2 and IL-7 for the common gamma chain providing an intermediate proliferation rate instead of an additive or synergistic effect. Supporting this hypothesis, we have previously shown that blocking the IL-2 receptor alpha with a monoclonal antibody increase the availability of the common gamma chain and render conventional T cells more responsive to IL-7 (16). To have a better insight into this competition mechanism, we performed experiments to determine the binding of IL-2 FITC when IL-7 was present in the culture or the binding of IL-7 FITC when IL-2 was present in the culture. Results showed that in the presence of increasing concentration of IL-7 the binding of IL-2 to Treg progressively decreased with a reduction of 70% in the presence of 10⁴ pg/ml of IL-7 (Figure 2A). On the other hand, the effect of IL-2 on the binding of IL-7 FITC to Treg was less pronounced with a reduction of 45% in the presence of 100 UI of IL-2 (Figure 2B). To formally prove that IL-7 interferes with the association of CD25 and CD132 (and possibly CD122) to form the high affinity IL-2 receptor, we developed a co-precipitation assay

to measure receptor association by flow cytometry (Figure 2C) similar to the one we previously shown (16). Treg pre-treated with increasing concentrations of IL-7 showed a progressive reduction of the association of the CD25/CD132 complex, suggesting that IL-7 decreased the availability of CD132 to form the high affinity IL-2 receptor complex. Taken together these data indicate that it is possible to elicit a significant expansion of Treg using a combination of IL-7 and IL-2 and that Treg with a naïve and stem cell memory phenotype are the subset with the strongest responsiveness to IL-7. Based on these data we decided to expand sorted CD45RA⁺CD62L⁺ Treg in the presence of IL-7 and IL-2.

Expansion, phenotype and suppressive capacity of naïve Treg expanded with IL-2 or a combination of IL-2 and IL-7.

We then compared two methods for expansion of highly purified (Supplementary figure 2 A, B and C) sorted CD45RA⁺CD62L⁺ Treg (Treg-n/Treg-scm). A standard method (StM: anti CD3/CD28 microbeads at 1:1 ratio in the presence of 100UI of rhIL-2) was compared to the IL-7 method (IL-7M) similar to the StM but with the addition of 10ng/ml of rhIL-7. After 14 days Treg expanded more with the StM (fold expansion: 68, 61-93) than with the IL-7M (fold expansion: 49, 47-59, $P=0.021$) resulting in a 28% decrease in the final cell yield using the IL-7M (Figure 3A). Surface phenotype analysis showed that Treg expanded with the IL-7M were highly enriched in CD45RA⁺CD62L⁺CD95⁺ cells (Figure 2B). Dividing Treg into subsets according to the expression of CD45RA, CD62L and CD95 we observed that Treg expanded with the two methods showed a similar percentage (median, IQR) of Treg-n (StM 5.4, 3.4-8.1 vs IL-7M 3.5, 2.4-5.5, $p=0.234$), Treg-cm (StM 39, 29.7-45.7 vs IL-7M 46, 40.1-57, $p=0.458$) and Treg-emra (StM 1.1, 0.8-1.2 vs IL-7M 1, 0.3-1.2, $p=0.88$) after 14 days of expansion (Figure 2C). Treg expanded with the IL-7M were highly enriched in Treg-scm (StM 4.3, 2.6-6 vs IL-7M 23.2, 17.9-31, $p=0.0078$) and have less Treg-em (StM 51, 40.5-63.5 vs IL-7M 23.5, 11.7-39, $p=0.0375$) (Figure 2C). After 14 days Treg cultures were magnetically depleted of anti CD3/CD28 microbeads and cultured for additional 3 days in the absence of cytokines (resting period). After resting expanded Treg showed a preservation of the relative percentages of Treg subsets after resting. Treg expanded with the StM or the IL-7M methods were tested in a suppression assay against CD8⁺ T cells, showing a significant reduction in the suppressive activity of Treg expanded with the IL-7M compared to Treg expanded with the StM (Figure 2D). Since we have shown that IL-7 can reduce the Treg suppressive activity but the suppressive function is re-established once IL-7 is removed, we tested Treg after a 3 day resting period, which resulted in a full recovery of the suppressive function after removal of IL-7. To determine whether the kinetic of proliferation can impact on the differences in the phenotype of expanded Treg we analyzed the phenotype of proliferating Treg according the dilution of CFSE after 5 days of expansion. Treg expanded with the StM showed a homogeneous proliferation pattern in which the large majority of Treg performed several cell cycles and lost the expression of CD45RA (Figure 3E and 3F). Treg expanded with the IL-7M can be divided in two groups, of which one performed multiple cell cycles and lost CD45RA, and a second one which performed a limited number of cell cycles and preserves the expression of CD45RA. Sorted Treg that performed more than two cell cycles from both the StM and the IL-7M were committed to differentiate predominantly into Treg-cm and Treg-em (Figure 3G). On the other hand, sorted Treg that performed less than two cell cycles from both the StM and the IL-7M were committed to differentiate into Treg-n and Treg-scm. These results suggest that a population of slowly proliferating Treg that is predominant in the IL-7M (but very scarce in the StM) is responsible for the abundance of Treg with a stem cell memory phenotype found in the final Treg product after 14 days of culture. Taken together these data suggest that the presence of IL-7 reduces the proliferation rate (and the final cell yield) but preserves a poorly differentiated phenotype. Moreover, the reduced suppressive function can be recovered after a brief culture in the absence of IL-7.

In vitro performances of naïve Treg expanded with the StM or the IL-7M

As expanded Treg were shown to undergo a rapid and substantial reduction once infused in patients, we aim to determine the in vitro performances of Treg expanded with the StM or the IL-7M. First, we determined parameters associated to the bio-energetic metabolism that can account for the metabolic fitness of expanded Treg. In vitro Treg expand in the presence of high glucose concentrations and availability of oxygen but these

two conditions are not always met in the human body, where glucose concentration is 5.5mM and oxygen availability is highly dependent on the anatomical localization, potentially affecting the Treg capacity to fulfill the metabolic needs once infused in patients. Treg expanded with the IL-7M showed an increased mitochondrial mass (MFI, IQR) compared to Treg expanded with the StM (StM 422, 240-743 vs IL-7M 1221, 1082-1427, $p=0.031$) as measured using the MitoTracker Green dye that accumulates in mitochondria regardless of mitochondrial membrane potential (Figure 4A). A similar mitochondrial membrane potential ($\Delta\psi$) was found in the two groups as measured using the MitoTracker® Deep Red FM dye (Figure 4B). To measure glucose uptake capacity we performed a dynamic assay by measuring the uptake of the glucose analog 2NBDG over a 50 minute time window. Data were analysed by comparing the differences in the area under the curve (AUC). Treg generated with the IL-7M showed a greater capacity to accumulate 2NBDG as compared to Treg generated with the StM (AUC; StM 3.5×10^6 vs IL-7M 4.8×10^6) (Figure 4C). Corroborating these findings, Treg expanded with the IL-7M produced more lactate than Treg expanded with the StM (StM 182, 133-233 vs IL-7M 367, 295-430, $p=0.03$) (Figure 4D) suggesting an increased glycolytic rate. These immuno-metabolic signatures are preserved after a resting period of three days in the absence of cytokines and antiCD3CD28 microbeads even if the intensity of signals are reduced accordingly to a lack of growth factors (Suppl Figure 3A). To test the performances of Treg in stress conditions, we performed experiments in which expanded Treg were cultured without cytokines and antiCD3/CD28 microbeads replicating the environmental change that Treg will face once infused in patients. The number of Treg expanded with the IL-7M declined less sharply than the number of Treg expanded with the StM. After three weeks of culture a significantly higher number of Treg expanded with the IL-7M was still viable (absolute number $\times 10^3$; StM: 46, 26-71 vs IL-7M 338, 272-386, $p=0.031$) (Figure 4E). Notably, when cytokines and beads were added back to the culture, Treg expanded with the IL-7M were also able to recover and re-expand after a second challenge. We next challenged expanded Treg with a direct pro-apoptotic stimulus using an agonistic anti-fas IgM antibody. We found that the percentage of Treg undergoing apoptosis was significantly lower in Treg expanded with the IL-7M (% annexin V+: StM 69.8, 41.9-78.2 vs IL-7M, 35.4, 21.6-39.6, $p=0.018$) (Figure 4F). As IL-7 was shown to up-regulate the anti-apoptotic molecule Bcl-2, we measured Bcl-2 in Treg expanded with the two methods and compared to Treg-n before expansion (Figure 4G). While expanded Treg showed an up-regulation of Bcl-2 compared to the non-expanded Treg-n, Treg expanded with the IL-7M display a significantly higher expression of Bcl-2 (MFI: StM 464, 320-684 vs IL-7M 892, 510-1142, $p=0.043$). Finally we measured the length of telomeric DNA after expansion as telomere erosion is associated with cell senescence. As compared to Treg-n, we observed a substantial reduction in telomere length in expanded Treg with both methods, but Treg expanded with the IL-7M showed significant preservation of telomeric DNA compared to Treg expanded with the StM (MFI: StM 729, 456-880 vs IL-7M 1356, 1095-1712, $p=0.0158$).

Homing and survival of Treg expanded with the StM or the IL-7M in NSG mice

To determine differences in the in vivo homing capacity and survival, Treg were injected in NSG mice. To determine homing expanded and IVI-sense-labelled Treg generated with the StM or the IL-7M were injected in the tail vein of NSG mice and monitored for 48 hours using the IVIS technology. After injection of Treg expanded with the StM, we observed an increased signal in the bladder (Figure 5A). As the labeling dye is excreted with urine we interpreted this signal as a consequence of increased cell death and release of free dye that accumulate in the bladder. After injection of Treg expanded with the IL-7M we noticed an increased signal in the femurs of mice, indicating an improved home capacity to the bone marrow. Corroborating these findings we observed an increased expression of CXCR4 in Treg expanded with the IL-7M (Figure 5B). Treg expanded with the IL-7M showed also an increased expression of CCR7 (also in line with a poorly differentiated phenotype), a similar but very low expression of CCR2 and a reduced expression of CCR9, indicating other possible differences in the migratory pattern of Treg expanded with the StM or the IL-7M. We next determined the overall persistence of Treg expanded with the StM or the IL-7M by injecting Treg intra-peritoneally in NSG mice. To distinguish injected Treg from allogenic PBMC used as feeder cells in the model we selected HLA-A*0201 Treg for expansion and track them in NSG mice using a anti HLA-A*0201 monoclonal antibody (Figure 5C). Treg expanded using the StM or the IL-7M protocol were co-injected in the same mice and labeled with CFSE and PBSE respectively for tracking. In peripheral blood we were able

to detect Treg expanded with the IL-7M for approximately 11 days whereas Treg expanded with the StM were detectable only until day 5 (Figure 5D). After 14 days from injection (with undetectable circulating Treg) mice were sacrificed and spleens were removed to detect the presence of residual Treg. We found that a significantly higher percentage of Treg expanded with the IL-7M compared to Treg expanded with the StM were present in the spleen of mice, consistent with a prolonged capacity to survive in vivo (Figure 5E).

Discussion

Adoptive Treg transfer with the aim to improve the control of immune responses and re-establish peripheral tolerance holds therapeutic promises in transplantation, GVHD, and autoimmune diseases. In a future perspective, antigen expanded as well as TCR engineered or CAR Treg will rapidly become available alternatives to polyclonal Treg. The clinical experience however has shown some issues that may impact the therapeutic value of this approach. Specifically, the need of a high number of Treg for adoptive therapy has been fulfilled with robust in vitro expansion protocols, but expanded Treg have shown fragilities and a limited capacity to persist in patients. Moreover, there is a need of measurable biological parameters that, beside the mere cell yield, can predict the capacity of expanded Treg to adapt to an in vivo environment in which competition with other cells for growth factors and nutrients generate a selective pressure. We report that Treg can be expanded using a combination of IL-7 and IL-2 with several advantages in term of resistance to apoptosis and stress and maintenance of a poorly differentiated phenotype. These findings are relevant to the improvements of Treg expansion protocols for adoptive Treg therapy and to overcome some issues related to the long-term persistence of Treg once infused in patients.

In vitro expansion of Treg for adoptive transfer traditionally rely on the use of high doses of IL-2 (17) (18) (19). The final Treg product is composed by a large majority of Treg with a short life-span in vivo and a long-lived subset that can persist for over a year (8). Based on studies performed mainly on conventional T cell we hypothesized the use of homeostatic cytokines for Treg expansion. Homeostatic cytokines such as IL-7 induces conventional T cell expansion (20) and provide anti-apoptotic signals (21). However, their use in Treg expansion has not been fully explored based on the low expression of the IL-7 receptor alpha chain, and assuming that Treg do not respond to IL-7.

Treg are phenotypically defined as $CD4^+CD25^{high}FOXP3^+$ T cells and sorted as $CD4^+CD25^{high}CD127^{low}$ T cells for in vitro expansion (14). Compared to circulating B cells that lack the expression of CD127 (15) we found a low but significant expression of CD127 that is sufficient to trigger significant STAT5 phosphorylation upon stimulation with 10ng/ml of IL-7. We previously reported that Treg are responsive to IL-7, even though at higher concentrations than conventional T cells (12). At physiological IL-7 concentrations of 2-8 pg/ml (22) Treg are not likely to receive significant signals, but when IL-7 is used at high concentration for in vitro expansion can elicit a robust response to IL-7. Importantly, we reported that in patients experiencing lymphopenia in which IL-7 is present at supra-physiological concentrations it may contribute to their homeostasis and proliferation to reconstitute the depleted Treg compartment (13). The response to IL-7 was higher in Treg with a naïve or memory stem T cell phenotype and decline with the progression of differentiation into central-memory and effector-memory subsets. The responsiveness was not strictly related to changes in the expression of CD127, suggesting that other unidentified factors may regulate STAT-5 phosphorylation beside the mere expression of the receptor. High IL-7 responsiveness of $CD45RA^+CD62L^+$ Treg was one reason why we decided to sort $CD45RA^+CD62L^+$ for expansion. The other reason was to obtain a Treg product with an immature phenotype that apparently are the cells that survive longer in patients (8). Since IL-7 per se was not sufficient to trigger significant Treg expansion we combined IL-7 to IL-2. Surprisingly, we did not observed an additive or synergistic effect but instead the presence of IL-7 reduced the proliferation rate induced by IL-2. We experimentally proved that in the presence of IL-7 the formation of the high affinity IL-7 receptor engage a significant amount of the common- γ chain (CD132), reducing the ability of IL-2 to form the high affinity IL-2 receptor with CD25, CD122 and CD132. We have previously shown that competition of CD127 and CD25 for CD132 can occur in conventional T cells (16). Accordingly, expansion of $CD45RA^+CD62L^+$ Treg with the IL-7M resulted in a reduced final cell yield as well as a different surface phenotype in which a significant higher proportion of Treg display a $CD45RA^+CD62L^+CD95^+$ phenotype.

This phenotype is reminiscent of that of conventional memory stem T cells (23) and, to the best of our knowledge Treg with a stem cell memory phenotype have never been described before. Further studies are needed to clarify whether a subset with memory stem cell function exist also in the Treg compartment with characteristics of self-renewal and the capacity to generate the full phenotypic diversity of Treg subsets. An increased expression of CD95 was found in expanded Treg from cord-blood as compared to expanded Treg from peripheral blood (24) that also contain an increased proportion of CD45RA⁺CD62L⁺ Treg. Such phenotypically immature Treg population obtained with the IL-7M also showed relevant differences in terms of metabolic machinery and survival capacity. An improved metabolic fitness in terms of mitochondrial mass and capacity to uptake glucose can advantage Treg expanded with the IL-7M once infused in patients. Compared to conventional T cells, Treg cells are less reliant on glycolysis and use mitochondrial metabolism and oxidative phosphorylation (OXPHOS) for energy production (25). In vitro studies revealed that Foxp3 is directly responsible in reprogramming T cell metabolism by suppressing glycolysis and enhancing OXPHOS (26)(27). Elevated glycolysis may be detrimental to Treg cell induction and suppressive function and deletion of HIF-1a, a transcription factor that can promote glycolysis, leads to increased Foxp3 induction (28). However, also Treg needs glycolysis to support some processes such as proliferation (29) and migration (30) when a high amount of ATP and metabolic intermediates are needed. It is reasonable to speculate that Treg cells precisely balance cellular glucose consumption, when glycolysis increases Treg cell proliferation and expansion, but this activity is balanced OXPHOS to maintain lineage stability and suppressive activity. Treg obtained with the IL-7M showed signs of both increased glycolysis (2NBDG uptake and lactate production) but also an increased mitochondrial mass. Reduction of glycolysis after a few days of resting corresponded, in our study, to a recovery of the Treg suppressive capacity. An important issue is also that Treg adoptive transfer implicates moving Treg from in vitro culture conditions with high glucose, oxygen and intense cytokine and TCR/CD28 signaling to an in vivo environment where these factors are reduced or completely missing. Therefore, a improved metabolic machinery can be helpful to Treg to adapt to changing conditions and survive in vivo overcoming substrate and growth factor signaling restrictions. We showed indeed an increased survival capacity of Treg both after growth factor deprivation but also in response to direct apoptotic stimuli possibly. Naïve Treg do not express CD95 and become susceptible to fas ligand mediated apoptosis only when start expressing CD95 upon stimulation (31). Increased resistance to fas ligand mediated apoptosis in Treg expanded with the IL-7M can be due to the increased expression of the anti-apoptotic molecule Bcl-2, which is presumably induced by the presence of IL-7 (21). We also observed a reduced telomere shortening in Treg expanded with the IL-7M. Telomere length is regulated by telomere erosion during cell division, and by the activity of telomerase which is negligible in T cells (32). Preservation of telomere length during IL-7 mediated T cell expansion has been reported (32), however in our Treg expansion model this could also be due to the reduced rate of expansion of Treg with the IL-7M. Preliminary data indicate an increased capacity of Treg expanded with the IL-7M to survive in NSG mice as well as a distribution pattern that includes migration to the bone marrow. While these observations require confirmation in relevant disease models to determine whether the increased persistence is associated to an improved therapeutic effect, our data prompted us to further characterized Treg expanded with the IL-7M. It has to be determined whether a lower number of phenotypically immature Treg but also with better performances can increase the therapeutic value as compared to a higher number of phenotypically mature Treg with fragilities in terms of resistance to stress and apoptosis.

We therefore suggest that addition of IL-7 during expansion improves the performances of Treg despite a lower final cell yield and a (reversible) reduction of suppressive function. Our expansion model needs to be further explored as a potential improvement of current Treg expansion protocols based on IL-2. The fragility of Treg used for adoptive transfer represents an issue that need to be resolved in order to increase the efficacy of this promising immune-therapy.

Methods

Cell isolation, purification, and FACS analysis

Sodium-heparinized peripheral venous blood samples were kindly provided by the Immunohematology and

Transfusion Medicine Service of San Raffaele hospital, Milan, Italy and were derived from healthy donors who donate blood. Highly purified (98%) naive Treg were isolated using the FACS Aria II according to the expression of CD4-PB (Clone SK3), CD25-APC-Cy7 clone (eBioDDR5), CD127-PE-CY7 (clone HIL-7R-M21), CD45RA-PE-Cy5 (clone HI-100), CD62L-PE (clone DREG-56), and CD95-APC (clone DX2) all from BD Biosciences (Supplemental figure 1). The same antibodies were used for FACS analysis and with the additional staining for FOXP3-AF488 (clone PCH101, from eBioscience). According to the experimental needs STAT-5 phosphorylation was measured using anti STAT5 pY694 Alexa Fluor 647 (clone 47, BD Biosciences) and Bcl-2 was detected using anti Bcl-2 APC (clone Bcl-2/100, BD Biosciences). Telomere length was measured using a fluorescein conjugated PNA probe kit from DAKO. Apoptosis was measured using an annexin V- propidium iodide staining kit from BD Biosciences.

IL-7 and IL-2 binding to receptors and association of CD25 and CD132

IL-7 and IL-2 binding to their respective receptors on the cell surface were measured using the IL-7 or the IL-2 Fluorokine kit according to manufacturer instructions (R&D System). Tregs were suspended in PBS at 4×10^6 cells/mL in the presence of rhIL-2 (Bio-Techne) or rhIL-7 (R&D Systems) at concentrations as indicated in figure 2 for 15 minutes at 37°C and incubated 1 µg/ml of biotinylated IL-7 or IL-2 for 20 minutes on ice. Avidin-FITC was then added for an additional 30 min. Finally, cells were washed in PBS and analyzed by FACS. To determine the association of IL-2 with CD25 and CD132 Treg were suspended in X-vivo 15 without serum and incubated with or without IL-7 at concentrations indicated in figure 2 for 15 min at 37°C. Treg were washed twice in PBS and cell pellets were frozen at -80°C, subsequently thawed and lysed in 50 mM Hepes, 150 mM NaCl, 15 mM MgCl₂, 1 mM EDTA, 10% glycerol and 1% Triton X-100. For the detection of association of CD25 and CD132, cell lysates were incubated with anti-CD25 PE (mouse IgG1, clone M-A251) on ice for 20 min. Anti-mouse IgG1,j-coupled beads (Comp beads, BD-Pharmingen) or uncoupled beads as control were added to the lysates for 20 min on ice to bind the anti-CD25 complex to the beads. Association of CD132 with CD25 was detected after washing beads in PBS containing BSA and incubation with biotinylated anti-CD132 (rat IgG2,j, clone TUGh4) for 20 min on ice. Beads were then incubated with streptavidin-APC for 20 min at room temperature, washed and analyzed by flow cytometry.

***In vitro* expansion protocols**

Sorted naïve Treg were plated at 5×10^4 /ml in 96-well U bottom plate (Costar) with X-VIVO 15 medium (Lonza, catalog n°. 04-418Q) containing 5% heat inactivated pooled bovine serum (Sigma-Aldrich). In the standard method (StM) culture medium was supplemented with Dynabeads human T activator CD3/CD28 (ThermoFisher) at a 1:1 bead/cell ratio, and 100 UI/ml rhIL-2 (Bio-Techne). In the IL-7 method (IL-7M), culture medium of the StM was supplemented also with rhIL-7 (R&D Systems) at 10 ng/ml. Treg were cultured for 14 days and the culture medium and supplements were replaced at day 7 and 11.

Treg homing and persistence in NSG mice

Homing of Treg was evaluated using the IVIS technology. Treg expanded with StM or the IL-7M were labelled with VivoTrack-680 (Perkin Elmer) and 2×10^6 of labelled cells were injected intravenously into NSG mice. In vivo monitoring of labelled cells was done in a Lumina II IVIS imaging system (Caliper Life Science), by recording fluorescence at an excitation wavelength of 675 nm and emission 582 filter of 690-770 nm. Data analysis was done using the Living Image software v. 4.2 (Caliper Life Sciences). To determine persistence of Treg in NSG mice, Treg expanded with the StM were labelled with CFSE and Treg expanded with the IL-7M were labelled with PBSE, and subsequently co-injected intravenously in NSG mice along with 10^6 allogenic PBMC used as feeder cells. Treg were generated from an HLA-A*0201 donor and anti HLA-A*0201-PE (clone BB7.2, Thermo Fisher) was used to distinguish Treg from feeder PBMC. Blood samples were collected to detect the presence of Treg generated with the two expansion protocols. After 14 days mice were sacrificed and the spleen was collected to detect the presence of Treg generated with the two expansion protocols.

Suppression assay

After expansion Treg were stained with eFluor 670 Cell Proliferation Dye and co-cultured with CFSE stai-

ned, FACS sorted, allogenic CD8+ T cells at 1:1, 1:2, 1:4, 1:8 and 1:0 Tcell:Treg ratios in the presence of anti CD3/CD28 beads at 1:10 cell/bead ratio. Cell culture was harvested at day 5 and the percentage of proliferating (CFSE diluted) T cells was calculated. The percentage of suppression was calculated as $100 - [100 \times (\text{percentage of proliferating cells with Treg present}) / (\text{percentage of proliferating cells without Treg present})]$.

T-Cell Bio-energetic Profile

Surrogate markers of oxidative phosphorylation and glycolysis were measured by flow cytometry using the following reagents according to the manufacturer's instructions. MitoTracker Green FM and MitoTracker Red FM were from Invitrogen-Molecular Probes. 2-(N-(7-nitrobenz-2-oxa-,1,3-diazol-4-yl)amino)-2-deoxyglucose (2NBDG) was from Life Technologies. L(+)lactate concentration was measured in the supernatants with a Lactate Assay Kit (Sigma-Aldrich).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Flow cytometry data were calculated as median fluorescence intensity (MFI) and subpopulations as percentage of positive cells. Proliferation was calculated as percentage of cells that diluted the fluorescent dye CFSE (%CFSEdim). Data were presented as median and interquartile range (IQR), and the Wilcoxon matched-pairs test was used for comparisons. A two-tailed *P* value of 0.05 was considered significant.

Study Approval

Blood donors signed an informed consent in accordance with the D.M. November 2nd 2015 entitled "Provisions relating to the quality and safety requirements of blood components". In accordance with the ministerial provisions and the IOG 364 institutional procedure "Request and delivery of buffy coats for research purposes", it is not possible to retrieve any type of information (gender, age, HLA typing) of donors. The animal study protocol was approved by the local animal ethics committee of San Raffaele Hospital (IACUC n 532/2022-PR, approved September 12, 2022).

Acknowledgements

Author contributions . I.C., J.F., A.F., and C.D.D. designed and performed the experiments, analyzed data, and contributed to write the paper. L.P. and P.M. designed the study, supervised the work, analyzed data, and wrote the paper. P.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data availability statement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding/financial support . This study was supported by the EFSD/JDRF/Lilly European Program in Type 1 Diabetes Research, and by grants from Fondazione Italiana Diabete (FID), and the Juvenile Diabetes Research Foundation JDRF (2-SRA-2021-1002-S-B)

References

1. Sakaguchi S, et al. Regulatory T Cells and Immune Tolerance. *Cell* . 2008;133(5):775-787.
2. Plitas G, Rudensky AY. Regulatory T cells: Differentiation and function. *Cancer Immunol Res* . 2016;4(9):721-725.
3. Atif M, et al. Regulatory T cells in solid organ transplantation. *Clin Transl Immunol*. 2020;9(2):1-15.
4. Guo WW, et al. Regulatory T Cells in GVHD Therapy. *Front Immunol* . 2021;12.
5. Esensten JH, et al. Regulatory T-cell therapy for autoimmune and autoinflammatory diseases: The next frontier. *J Allergy Clin Immunol* . 2018;142(6):1710-1718.

6. Raffin C, Vo LT, Bluestone JA. T reg cell-based therapies : challenges and perspectives. *Nat Rev Immunol* . 2021;20(3):158-172.
7. Mohseni YR, et al. The Future of Regulatory T Cell Therapy: Promises and Challenges of Implementing CAR Technology. *Front Immunol* . 2020;11:1-13.
8. Bluestone JA, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med* . 2015;7(315).
9. Seddiki N, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* . 2006;203(7):1693-1700.
10. Hoffmann P, et al. Large-scale in vitro expansion of polyclonal human CD4+CD25 high regulatory T cells. *Blood* . 2004;104(3):895-903.
11. Vignali D, et al. Detection and characterization of CD8 + autoreactive memory stem T cells in patients with type 1 diabetes. *Diabetes* . 2018;67:936-945.
12. Heninger A-K, et al. IL-7 abrogates suppressive activity of human CD4+ CD25 + FOXP3+ regulatory T cells and allows expansion of alloreactive and autoreactive T cells. *J Immunol* . 2012;189(12). 5649-5658
13. Vignali D, et al. IL-7 Mediated Homeostatic Expansion of Human CD4+CD25+FOXP3+ Regulatory T Cells After Depletion With Anti-CD25 Monoclonal Antibody. *Transplantation* . 2016;100(9). 1853-1861
14. Liu W, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* . 2006;203(7):1701-1711. 15. Ryan BDH, et al. Expression of interleukin-7 receptor by lineage-negative human bone marrow progenitors with enhanced lymphoid proliferative potential and B-lineage differentiation capacity. *Blood* . 1997.89(3):929-940
16. Monti P, et al. Disengaging the IL-2 receptor with daclizumab enhances IL-7-mediated proliferation of CD4+ and CD8+ T Cells. *Am J Transplant* . 2009;9(12).2727-2735
17. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3 + regulatory T cells. *Blood* . 2005;105(12):4743-4748.
18. Putnam AL, et al. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* . 2009;58(3):652-662.
19. MacDonald KN, Piret JM, Levings MK. Methods to manufacture regulatory T cells for cell therapy. *Clin Exp Immunol* . 2019;197(1):52-63.
20. Tan JT, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A* . 2001;98(15):8732-8737.
21. Chetoui N, et al. Interleukin-7 promotes the survival of human CD4+ effector/memory T cells by up-regulating Bcl-2 proteins and activating the JAK/STAT signalling pathway. *Immunology* . 2010;130(3):418-426.
22. Carrett F, Surh CD. IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. *Semin Immunol* . 2012;24(3):209-217.
23. Gattinoni L, et al. A human memory T cell subset with stem cell-like properties. *Nat Med*. 2011;17(10):1290-1297.
24. Motwani K, et al. Human Regulatory T Cells From Umbilical Cord Blood Display Increased Repertoire Diversity and Lineage Stability Relative to Adult Peripheral Blood. *Front Immunol* . 2020;11:1-17.
25. Procaccini C, et al. An Oscillatory Switch in mTOR Kinase Activity Sets Regulatory T Cell Responsiveness. *Immunity* . 2010;33(6):929-941.

26. Angelin A, et al. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metab* . 2017;25(6):1282-1293.e7.
27. Garriets VA et al. Foxp3 and Toll-Like Receptor Signaling Balance Treg Cell Anabolic Metabolism for Suppression. *Nat Immunol* . 2016;17(12):1459-1466
28. Shi, et al. HIF1 α -dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med* . 2011;208(7):1367-1376.
29. Pacella I, et al. Fatty acid metabolism complements glycolysis in th selective regulatory t cell expansion during tumor growth. *Proc Natl Acad Sci U S A*. 2018;115(28):E6546-E6555.
30. Kishore M, et al. Regulatory T Cell Migration Is Dependent on Glucokinase-Mediated Glycolysis. *Immunity* . 2017;47(5):875-889
31. Fritzsching B, et al. Naive regulatory T cells: A novel subpopulation defined by resistance toward CD95L-mediated cell death. *Blood* . 2006;108(10):3371-3378.
32. Patrick MS, Cheng NL, Kim J, et al. Human T cell differentiation negatively regulates telomerase expression resulting in reduced activation-induced proliferation and survival. *Front Immunol* . 2019;10:1-11
1. Sakaguchi S, et al. Regulatory T Cells and Immune Tolerance. *Cell* 2008;133(5):775–787.
2. Plitas G, Rudensky AY. Regulatory T cells: Differentiation and function. *Cancer Immunol. Res.* 2016;4(9):721–725.
3. Atif M, et al. Regulatory T cells in solid organ transplantation. *Clin. Transl. Immunol.* 2020;9(2):1–15.
4. Guo WW, et al. Regulatory T Cells in GVHD Therapy. *Front. Immunol.* 2021;12(June). doi:10.3389/fimmu.2021.697854
5. Esensten JH, et al. Regulatory T-cell therapy for autoimmune and autoinflammatory diseases: The next frontier. *J. Allergy Clin. Immunol.* 2018;142(6):1710–1718.
6. Raffin C, Vo LT, Bluestone JA. T reg cell-based therapies : challenges and perspectives 2021;20(3):158–172.
7. Mohseni YR, et al. The Future of Regulatory T Cell Therapy: Promises and Challenges of Implementing CAR Technology. *Front. Immunol.* 2020;11(July):1–13.
8. Bluestone JA, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells 2015;7(315).
9. Seddiki N, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 2006;203(7):1693–1700.
10. Hoffmann P, et al. Large-scale in vitro expansion of polyclonal human CD4+CD25 high regulatory T cells. *Blood* 2004;104(3):895–903.
11. Vignali D, et al. Detection and characterization of CD8 + autoreactive memory stem T cells in patients with type 1 diabetes. *Diabetes* 2018;67(May):936–945.
12. Heninger A-K, et al. IL-7 abrogates suppressive activity of human CD4+ CD25 + FOXP3+ regulatory T cells and allows expansion of alloreactive and autoreactive T cells. *J. Immunol.* 2012;189(12). doi:10.4049/jimmunol.1201286
13. Vignali D, et al. IL-7 Mediated Homeostatic Expansion of Human CD4+CD25+FOXP3+ Regulatory T Cells After Depletion With Anti-CD25 Monoclonal Antibody 2016;100(9). doi:10.1097/TP.0000000000001276
14. Liu W, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* 2006;203(7):1701–1711.
15. Ryan BDH, et al. and B-Lineage Differentiation Capacity 1997;

16. Monti P, et al. Disengaging the IL-2 receptor with daclizumab enhances IL-7-mediated proliferation of CD4⁺ and CD8⁺ T Cells. *Am. J. Transplant.* 2009;9(12). doi:10.1111/j.1600-6143.2009.02825.x
17. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4⁺CD25⁺FoxP3⁺ regulatory T cells. *Blood* 2005;105(12):4743–4748.
18. Putnam AL, et al. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* 2009;58(3):652–662.
19. MacDonald KN, Piret JM, Levings MK. Methods to manufacture regulatory T cells for cell therapy. *Clin. Exp. Immunol.* 2019;197(1):52–63.
20. Tan JT, et al. IL-7 is critical for homeostatic proliferation and survival of naïve T cells. *Proc. Natl. Acad. Sci. U. S. A.* 2001;98(15):8732–8737.
21. Chetoui N, et al. Interleukin-7 promotes the survival of human CD4⁺ effector/memory T cells by up-regulating Bcl-2 proteins and activating the JAK/STAT signalling pathway. *Immunology* 2010;130(3):418–426.
22. Manuscript A. NIH Public Access 2013;24(3):209–217.
23. Gattinoni L, et al. A human memory T cell subset with stem cell-like properties. *Nat. Med.* 2011;17(10):1290–1297.
24. Motwani K, et al. Human Regulatory T Cells From Umbilical Cord Blood Display Increased Repertoire Diversity and Lineage Stability Relative to Adult Peripheral Blood. *Front. Immunol.* 2020;11(April):1–17.
25. Procaccini C, et al. An Oscillatory Switch in mTOR Kinase Activity Sets Regulatory T Cell Responsiveness. *Immunity* 2010;33(6):929–941.
26. Angelin A, et al. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metab.* 2017;25(6):1282–1293.e7.
27. Issa F. Foxp3 and Toll-Like Receptor Signaling Balance Treg Cell Anabolic Metabolism for Suppression. *Transplantation* 2017;101(2):218.
28. Shi LZ, et al. HIF1 α -dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J. Exp. Med.* 2011;208(7):1367–1376.
29. Pacella I, et al. Fatty acid metabolism complements glycolysis in the selective regulatory t cell expansion during tumor growth. *Proc. Natl. Acad. Sci. U. S. A.* 2018;115(28):E6546–E6555.
30. Kishore M, et al. Regulatory T Cell Migration Is Dependent on Glucokinase-Mediated Glycolysis. *Immunity* 2017;47(5):875–889.e10.
31. Fritzsche B, et al. Naive regulatory T cells: A novel subpopulation defined by resistance toward CD95L-mediated cell death. *Blood* 2006;108(10):3371–3378.
32. Patrick MS, et al. Human T cell differentiation negatively regulates telomerase expression resulting in reduced activation-induced proliferation and survival. *Front. Immunol.* 2019;10(AUG):1–11.

Hosted file

image1.emf available at <https://authorea.com/users/619937/articles/644220-il-7-improves-the-fitness-of-regulatory-t-cells-for-adoptive-transfer>

Figure 1. Identification and responsiveness to IL-7 of circulating Treg subsets. (A) Representative FACS plots showing the gating strategy to identify Treg (CD4⁺CD25^{high}FOXP3⁺CD127^{low}) and naïve and memory subsets based on the expression of CD62L, CD45RA and CD95. (B) Graph shows percentage of each of Treg subset from 16 subjects (C) Representative FACS plot (left) showing the expression of CD127 in Treg subsets compared to conventional CD4⁺ T cells and B cells, and graph representing data from 6

subjects (right). **(D)** . Representative FACS plot (left) showing STAT-5 phosphorylation in Treg subsets compared to conventional CD4+ T cells and B cells after 10 minutes incubation with 10ng/ml of rh-IL-7 (left) measurements from 6 subjects (right). **(E)** Expansion curve of bulk Treg cultured with medium (Ctr), IL-2 (100 UI/ml), IL-7 (10ng/ml), and combination of IL-2 and IL-7. (n=3).

Hosted file

image2.emf available at <https://authorea.com/users/619937/articles/644220-il-7-improves-the-fitness-of-regulatory-t-cells-for-adoptive-transfer>

Figure 2. IL-2 receptor and IL-7 receptor competition. **(A)** FACS plot showing the binding of IL-2-FITC (or unlabeled IL-2) to the Treg surface in the absence of IL-7 (left). Graph showing the binding of IL-2-FITC in the presence of increasing concentrations of IL-7 (0-10⁶ pg/ml) (right). **(B)** FACS plot showing the binding of IL-7-FITC (or unlabeled IL-7) to the Treg surface in the absence of IL-2 (left). Graph showing the binding of IL-7-FITC in the presence of increasing concentrations of IL-2 (0-10⁴ UI/ml) (right). **(C)** Flow cytometric assay to measure the association of CD25 and CD132 using beads. CD127 was linked to antimouse IgG1-coupled beads through a mouse-IgG1-anti-CD25 PE complex. CD132 association was determined by a biotinylated anti-CD132 and streptavidin APC. FACS plots shows the fluorescence of CD132 APC on beads alone (left), beads incubated with cell lysate from Treg incubated in PBS (center) and beads incubated with 10⁴ pg/ml of IL-7. Graph shows the fluorescence of CD132 APC on beads incubated with increasing concentrations of IL-7 (0-10⁶ pg/ml).

Hosted file

image3.emf available at <https://authorea.com/users/619937/articles/644220-il-7-improves-the-fitness-of-regulatory-t-cells-for-adoptive-transfer>

Figure 3. Expansion of Treg in the presence of IL-7. **(A)** Fold expansion of naïve Treg after 14 days culture with the StM (aCD3CD28 beads + 100UI/ml rh IL-2) and the IL-7M (aCD3CD28 beads + 100UI/ml rh IL-2+ 10ng/ml rhIL-7) (n=9) **(B)** Representative FACS plots showing the phenotype of Treg after expansion with the StM or the IL-7M. **(C)** Graph shows the percentage of Treg subsets after 14 days expansion with the StM or the IL-7M and at day 17 after resting (medium only) (n=8) **(D)** Suppression assay of Treg after expansion with the StM or the IL-7M after 14 days expansion and at day 17 after resting (medium only) (n=3). **(E)** CFSE dilution assay showing the number of cell cycles performed by Treg in the first 5 days of expansion with the StM or the IL-7M **(F)** Graph shows the percentage of cells in each cycle after 5 days. **(G)** Graph shows the percentage of cells displaying T naïve (Tn, CD45RA⁺CD62L⁺CD95⁻), T stem cell memory (Tscm, CD45RA⁺CD62L⁺CD95⁺), T central memory (Tcm, CD45RA⁻CD62L⁺CD95⁺), T effector memory (tem, CD45RA⁻CD62L⁻CD95⁺) phenotype.

Hosted file

image4.emf available at <https://authorea.com/users/619937/articles/644220-il-7-improves-the-fitness-of-regulatory-t-cells-for-adoptive-transfer>

Figure 4 . Bio-energetic profile, apoptosis and telomere length of Treg expanded in the presence of IL-7. **(A)** Mitochondrial mass and **(B)** mitochondrial $\Delta\psi_m$ of Treg expanded with the StM or the IL-7M. **(C)** Dynamic measurement of 2NBDG uptake in Treg expanded with the StM or the IL-7M **(D)** Graph shows lactate concentration (mmol/l) in the supernatant of Treg expanded with the StM or the IL-7M **(E)** Treg expanded with the StM or the IL-7M were cultured for three weeks in medium without aCD3CD28 beads nor cytokines. The absolute number of live cell is reported in the graph. After three weeks, remaining Treg were re-stimulated with aCD3CD28 beads and 100 UI rhIL-2. **(F)** Representative FACS plot (left) of annexin V and propidium iodide staining in Treg expanded with the StM or the IL-7M and cultured with an agonistic anti-fas monoclonal antibody. Graph representing four independent experiments is shown on the right. **(G)** Representative FACS plot (left) showing Bcl-2 expression in Treg expanded with the StM or the IL-7M and in naïve Treg after sorting (n=3). **(H)** Telomere length measure by flow cytometry using

a PNAC₃TA₂ probe was measured in Treg expanded with the StM or the IL-7M and in naïve Treg after sorting (n=4).

Hosted file

image5.emf available at <https://authorea.com/users/619937/articles/644220-il-7-improves-the-fitness-of-regulatory-t-cells-for-adoptive-transfer>

Figure 5. Homing and survival of Treg in NSG mice. (A) IVIS images of NSG mice injected with Treg generated with the StM of the IL-7M at time 0 (pre-injection), 12 hours and 48 hours after Treg injections. (B) FACS plots showing the expression of CXCR4, CCR7, CCR2 and CCR9 on Treg expanded with the StM (green line) of the IL-7M (blue line). (C) FACS plot showing the presence of HLA-A*0201⁺ Treg (feeder PBMC were HLA-A*0201⁻) in NSG mice at day 0 (pre-transfer), 2, 3, 5, 7, 9, and 11 (upper plots). Gated HLA-A*0201⁺Treg were detected according to labeling with CFSE (Treg StM) and PBSE (Treg IL-7M) (lower plots). (D) Graph shows the kinetic of Treg in NSG mice over an 11 day observation period. (E) Detection of Treg generated with the StM of the IL-7M at day 14 after transfer in the spleen of NSG mice.