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Increased CD127 expression on activated Foxp3⁺ CD4⁺ regulatory T cells

Federico Simonetta¹, Amel Chiali¹, Corinne Cordier², Alejandra Urrutia¹, Isabelle Girault¹, Stéphane Bloquet¹, Corinne Tanchot³, Christine Bourgeois^{1*}

¹ Régulation de la réponse immunitaire, infection VIH-1 et autoimmunité INSERM : U1012, Université Paris Sud - Paris XI, 63, rue Gabriel Péri, 94276 Le Kremlin-Bicêtre, FR

² Service commun de tri cellulaire IFR94, Faculté de Médecine, Site Necker Enfants-Malades, 142, rue de Vaugirard, 75015 Paris, FR

³ Différenciation et physiologie des lymphocytes T INSERM : U1020, Université Paris Descartes, Faculté de Médecine, Site Necker Enfants-Malades, 156, rue de Vaugirard, 75015 Paris, FR

* Correspondence should be addressed to: Christine Bourgeois <christine.bourgeois@u-psud.fr >

Abstract

SUMMARY

Regulatory T cells (Tregs) are commonly identified by CD25 (IL-2R α) surface expression and/or intracellular expression of Foxp3 transcription factor. In addition, Tregs are also characterized by low CD127 (IL-7R α) expression when compared to conventional T cells and their peripheral biology is considered essentially independent of IL-7. We further investigated CD127 expression on Tregs and we demonstrated differential CD127 expression depending on Treg subsets considered. Notably, we observed high CD127 expression on ICOS and CD103 expressing Treg subsets. Since these two markers reflect activation status, we addressed whether Treg activation modulated CD127 expression. We demonstrated that in contrast to conventional T cells, Tregs significantly up-regulated CD127 expression during in vitro and in vivo activation using adoptive transfer and contact dermatitis models. High CD127 expression on Tregs was also predominantly detected ex vivo in some specific sites, notably bone marrow and skin. Importantly, higher CD127 expression on Tregs correlated with higher phosphorylation of STAT5 upon IL-7 exposure. High CD127 expression on Tregs also provided survival advantage upon in vitro incubation with IL-7. We thus demonstrated low CD127 expression is not an intrinsic characteristic of Tregs and we identified activated Tregs as a potential target of endogenous or therapeutic IL-7.

Author Keywords Tregs, IL-7, CD127 (IL-7R α)

INTRODUCTION

CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Tregs) are a critical CD4 T cell subset involved in the control of immune homeostasis by preventing autoimmune diseases and regulating chronic inflammation [1, 2]. Tregs have been shown to exert suppressive activity by various mechanisms, notably by deprivation of IL-2 [3–5], inhibitory cytokine production (IL-10, TGF- β , IL-35), cytolytic function, metabolic disruption, modulation of APC function [6]. They are commonly identified by CD25 (IL-2R α) surface expression and/or intracellular expression of forkhead box P3 (Foxp3) transcription factor [7–9]. In addition to high CD25 expression, Tregs are also characterized by low CD127 (IL-7R α) expression when compared to conventional T cells [10–12]. Although CD127 expression and consequently IL-7/IL-7R signaling is crucial to most peripheral T cells, Tregs stand a remarkable exception and exhibit different cytokine requirement profile: IL-2 appears the main survival factor whereas IL-7 is thought to contribute moderately to Treg homeostasis [13–15]. High IL-2 requirement and low IL-7 influence on Tregs are in accordance with the high level of CD25 and low level of CD127 on Tregs. However, CD127 expression has been shown to be highly regulated on conventional T cells depending notably on IL-2 and IL-7 availability [16, 17] or TCR stimulation [18]. We thus questioned whether low CD127 expression was an intrinsic hallmark of Tregs or could be regulated by their activation status and/or environmental contexts. Firstly, we determined whether Tregs exhibited some heterogeneity in CD127 expression ex vivo. When addressing Tregs as subsets by introducing markers of Treg heterogeneity [19, 20], high CD127 expression correlated with the expression of markers such as CD103 and ICOS. Because these two markers have been described to identify activated Tregs [21–25], these results suggested that in contrast to conventional T cells activation, CD127 expression may be more prominent during Treg activation. To confirm this hypothesis, we developed different approaches. We detected higher CD127 expression on Tregs upon in vitro activation. Using adoptive transfer and contact dermatitis models, we confirmed in vivo that CD127 is highly expressed on activated Tregs both in lymphoid and non lymphoid tissues. Collectively, these data demonstrated high CD127 expression correlated with Treg activation. Finally, since Treg activation also occurs in peripheral tissues, we performed tissue specific analysis, revealing striking diversity in CD127 expression on Tregs depending on the sites considered. To formally identify activated Tregs as a preferential target of IL-7, we determined whether Tregs transduced intracellular signaling mediated by IL-7. We demonstrated that high levels of CD127 expression on Tregs correlated with increased IL-7 signal transduction. We finally confirmed the functional relevance of CD127 expression by demonstrating enhanced survival capacity of Tregs expressing high level of CD127 (CD127^{hi}) upon in vitro incubation with IL-7. Our results demonstrated that low CD127 expression is not an intrinsic characteristic of Tregs and that differential CD127 expression on Tregs depends on their localization and their activation status. They also revealed

differential regulation of CD127 expression among conventional and regulatory CD4 T cells upon activation, providing potential regulation of Treg biology depending on IL-7 availability.

RESULTS

ICOS and/or CD103 identify CD127 expressing Tregs ex vivo

Most CD4⁺ Foxp3⁺ regulatory T cells have been shown to exhibit low expression of CD127 compared to conventional CD4 and CD8 peripheral T cells both in humans and mice (11, 12). Regarding large phenotypic heterogeneity among Tregs, we questioned whether CD127 expression could differ depending on the Treg subsets considered. To obtain a comprehensive characterization of regulatory T cell phenotypic subsets, we determined on Foxp3⁺ CD4 T cells the expression of several markers discriminating activation status (CD62L, CD44, CD69), or known to be preferentially expressed on Tregs (GITR, CTLA-4) or representing co-stimulatory (CD28, ICOS, OX40, CD154, PD-1) and adhesion molecules (ICAM-1, CD103) (data not shown). Among the various molecules studied, we identified four markers for which the Treg population showed heterogeneity: in lymph nodes, CD62L was expressed by about half of Tregs (56% ± 6%); CD69 expression identified 34% ± 1% of Tregs; CD103 expressing Treg represented 21% ± 1% of the total Treg population and ICOS was expressed by 16% ± 2% of Tregs (Percentages are mean ± SD of a representative experiment as defined on a sample in Fig. 1.A). Prior to the analysis of CD127 on each subset, we confirmed the suppressive capacity of these respective subsets. CFSE-labeled sorted naïve CD4 T cells (CD62L⁺ CD25⁻ CD4⁺ T cells) were stimulated in vitro with anti-CD3 with or without Tregs and proliferation was assessed at day 3–4. As shown in Fig. 1B , all Treg subsets studied exerted a suppressive activity at least comparable with that of the whole CD4⁺ CD25⁺ population in a 1/1 Teff/Treg ratio. We next analyzed CD127 cell surface expression on total Tregs and Treg subset expressing or not CD62L, CD69, ICOS and CD103 (Fig. 1C). As previously published, total Tregs exhibited low CD127 expression compared to conventional CD4 T cells (CD127 MFI: 68 ± 12 and 302 ± 23 respectively). To note, level of CD127 expression on Tregs was significantly different from isotype control staining, confirming low but existing CD127 expression on Tregs. CD69 or CD62L expressing Tregs presented CD127 surface levels not significantly different from their negative counterparts. Importantly, CD103 or ICOS expressing Tregs exhibited significantly higher levels of CD127 compared to their negative counterparts (CD127 MFI: 111 ± 3 on CD103+ compared to 62 ± 14 on CD103-; 146 ± 30 on ICOS+ compared to 67 ± 12 on ICOS-). To determine whether ICOS and CD103 expressing Treg subsets were independent or partially overlapping, we analyzed the co-expression of ICOS and CD103 on Tregs and CD127 expression depending on ICOS and CD103 co-expression (Fig. 1D). CD103⁻ ICOS⁻ Tregs were predominant (73% ± 1%) and exhibited low CD127 expression. The three subsets identified by CD103 and/or ICOS expression (i.e. CD103 or ICOS single positive, CD103/ICOS double positive) were similarly represented (9% ± 1%, 7% ± 1% and 9% ± 1% of CD4⁺ Foxp3⁺ cells isolated from LN respectively) but exhibited very different CD127 profiles. Single positive cells for either ICOS or CD103 expressed CD127 levels comparable with those expressed by the whole Treg subset. Strikingly, levels of CD127 on CD103⁺ /ICOS⁺ cells were significantly higher when compared to total Tregs (CD127 MFI: 195 ± 12 on ICOS⁺ CD103⁺ Treg fraction compared to 68 ± 12 on total Tregs). We thus demonstrated that in LN, CD103/ICOS expression identified a fraction of CD4⁺ Foxp3⁺ T cells which exhibited high CD127 expression.

Tregs up-regulated CD127 during in vitro and in vivo activation

Since CD103 and ICOS have been described as Treg activation markers, we thus speculated that the CD127^{hi} Treg fraction could be more prominent during Treg activation. To directly address this assumption, we studied CD127 expression on Tregs upon in vitro and in vivo activation. Firstly, we determined CD127 expression simultaneously on Treg and conventional CD4 T cells following in vitro anti-CD3 stimulation of total CD4 T cells (Fig. 2A). At day 4, we confirmed increased ICOS expression during activation: ICOS expressing Tregs represented 92% ± 4% compared to 23% ± 2% ex vivo (Percentages expressed are mean ± SD of a representative experiment, as defined on one sample on Fig. 2A). To note, ICOS expression was also increased on non Treg CD4 T cells but to a lesser extent (59% ± 9% upon activation compared to 6% ± 1% ex vivo). 4 days after stimulation, CD127 expression was increased on Foxp3⁺ Tregs whereas CD127 down regulation was observed on some conventional Foxp3⁻ CD4 T cells (Fig. 2B). Interestingly, CD127 expression on activated Tregs was similar to CD127 expression on activated conventional cells (CD127 MFI: 354 ± 123 on activated Tregs compared to 248 ± 91 on activated conventional CD4 T cells) (Fig. 2C).

To confirm these data in vivo , we used a classical adoptive transfer model: sorted CD4⁺ CD25⁺ Tregs were co-injected with CD45.1⁺ naïve CD4 T cells into Rag deficient hosts. Analysis was performed six weeks after transfer and Treg activation was confirmed by CD103 and ICOS expression (data not shown). Transferred conventional Foxp3⁻ CD4 T cells expressed lower levels of CD127 when compared to conventional CD4 T cells isolated from WT mice (MFI: 196 ± 13 and 353 ± 45 respectively) (Fig. 3A, B). In contrast, activation of Tregs induced higher CD127 expression compared to ex vivo isolated Tregs (CD127 MFI: 154 ± 33 after transfer compared to 89 ± 10 ex vivo) (Fig. 3A, B). To determine whether higher CD127 expression relies on up regulation or expansion of preexisting activated Tregs, similar experiments were performed using “naive-like” Tregs (CD4⁺ CD25⁺ CD62L⁺ CD103⁻ CD69⁻ ICOS⁻). CD45.2⁺ transferred “naive-like” Tregs expressed higher levels of CD127 than ex vivo Tregs (CD127 MFI: 222 ± 22 after transfer compared to 89 ± 10 ex vivo) (Fig. 3C, D). High CD127 expression in “naive-like” Tregs supported up-regulation rather than expansion of a pre-existing CD127^{hi} Treg fraction.

We thus demonstrated that Tregs, while expressing low levels of CD127 at the steady state, significantly up-regulate this molecule upon *in vitro* and *in vivo* activation.

Activated Tregs expressed CD127 in a model of acute skin inflammation

Although adoptive transfer is a useful model to study Treg activation and suppressive capacity *in vivo*, it requires transfer into empty hosts, which may introduce some bias concerning CD127 expression analysis. It is well known that IL-7 availability directly interferes with CD127 expression at T cell surfaces [16, 17] and that IL-7 cytokine levels are increased in lymphopenic states [26, 27]. We wanted to confirm our results in a non-lymphopenic model of Treg activation: a model of acute skin inflammation. Ear skin exposure to DNFB induces a strong inflammatory response accompanied by ear swelling and activation of resident and recruited T cells [28]. Conventional and regulatory T cell activation was studied using ICOS expression. Indeed, ICOS is the most consistent marker for Treg activation whereas CD103 has been described to reflect tissue specific Treg homing. Moreover, being a shared activation marker for conventional and regulatory CD4 T cells, it also allows distinguishing activated conventional T cells. Analyses were performed six days after DNFB treatment. Inflammation was confirmed by significant increase in ear thickness (Fig. 4A) and increased CD4 T cell numbers in cervical lymph nodes draining the ear skin (Fig. 4B). In draining LN, a fraction of conventional CD4⁺ Foxp3⁻ cells underwent activation as revealed by ICOS up-regulation. Simultaneously, an important fraction of CD4⁺ Foxp3⁺ Tregs was activated and expressed ICOS (46 ± 6%). We next determined CD127 expression on activated regulatory and conventional CD4 T cells as identified by ICOS expression. As expected, ICOS expressing conventional CD4 T cells down-regulated CD127 at their surface. However, ICOS⁺ Tregs expressed higher levels of CD127 than their negative counterpart (Fig. 4C). To assess CD127 expression on CD4 T cell subsets at the site of inflammation, we analyzed lymphocytes infiltrating ear skin in both control and inflamed ear skin. In control mice, an important proportion of conventional and regulatory CD4 T cells infiltrating ear skin expressed ICOS (Fig. 4D) suggesting that resident CD4 T cells were in an activated state. During acute inflammation, a more diverse phenotype was observed both on conventional and regulatory T cells, revealing notably an increasing proportion of ICOS⁻ conventional and regulatory T cells. The rationale for the appearance of phenotypically less activated T cells (as assessed by low ICOS expression) is unclear but may reflect recruitment from peripheral blood or down regulation of ICOS on pre-existing resident T cells during the acute phase of inflammation. Nevertheless, we assessed CD127 expression on skin-infiltrating CD4 T cell subsets expressing or not ICOS, similarly to what we performed in draining LN. As shown in Fig. 4D, a fraction of ICOS⁺ conventional CD4 T cells down-regulated CD127 when compared to the ICOS⁻ population whereas a fraction of activated ICOS⁺ Tregs expressed higher levels of CD127 than their negative counterpart. The appearance of CD127^{lo} Tregs among ICOS⁺ cells in acute inflamed skin may also rely on recruitment or migration of peripheral blood derived T cells into inflamed sites but may also reflect different kinetics of expression of ICOS and CD127 on activated Tregs during the acute phase of inflammation. However, in both cases, high CD127 expression was predominantly observed on activated Tregs in ear skin. Collectively, these *in vivo* results demonstrated that upon activation, conventional CD4 T cells down-regulate CD127, whereas Tregs, which express low levels of CD127 at the steady state, strongly up-regulated this molecule. These results also confirmed that CD127 expression on activated Tregs can be equivalent to conventional T cells.

Different CD127 expression on Tregs depending on their tissue localization

Considering the high CD127 expression profile from skin resident Tregs in non immunized mice, we next analyzed CD127 expression on Tregs among various sites: lymph nodes (LN), spleen (SP), peripheral blood (PB), thymus, bone marrow (BM), peritoneal cavity (PC), intestine, lung, liver and skin. CD127 expression was determined on tissue resident Foxp3⁺ and conventional Foxp3⁻ CD4⁺ TCR⁺ cells and isotype staining was performed as control (Fig. 5A). CD127 expression profile on Tregs differed depending on the organs studied (Fig. 5B, C). Various proportions of CD127 high expressing Tregs were recovered, ranging from 10% ± 3% in the intestine to 82% ± 4% in the skin. Peripheral blood (15% ± 8%), lymph nodes (17% ± 4%), thymus (19% ± 1%), and spleen exhibited intermediate proportion of CD127 expressing Tregs (28% ± 6%). Strikingly, BM exhibited high percentage of CD127 expressing Tregs (64% ± 3%) (Fig. 5B). Similar results were obtained analyzing MFI (Fig. 5C). Highest levels of CD127 expression on Tregs were observed in BM and skin (CD127 MFI 312 ± 57 in BM and 383 ± 73 in skin compared to 85 ± 15 in LN). In these two organs, no concomitant up-regulation was observed on conventional T cells when compared to LN counterpart, suggesting a Treg restricted phenomenon. Our results demonstrated differential CD127 expression on Tregs depending on their localization and they confirmed that low CD127 expression was not an intrinsic characteristic of Tregs.

Higher levels of CD127 expression on Tregs correlated with increased IL-7 signal transduction

To assess whether CD127 expression on Tregs was associated with efficient IL-7 signal transduction, we examined STAT5 phosphorylation following IL-7 incubation *in vitro*. We used Foxp3-GFP reporter mice to identify Tregs; similar results were obtained using CD25 as a marker for Tregs identification. IL-2 treatment induced robust STAT5 phosphorylation in CD4⁺ Foxp3⁺ but not in conventional CD4⁺ Foxp3⁻ T cells (Fig 6A). IL-7 treatment induced significant increase in phospho-STAT5 in Tregs but to a lesser extent than observed in conventional CD4⁺ Foxp3⁻ cells, as previously described [13, 29, 30]. Importantly, a dose dependent effect was

detectable among Tregs but not among conventional T cells at all doses studied (Fig. 6A). We next studied STAT5 phosphorylation depending on CD127 expression on Tregs. As shown in Fig. 1.C , Tregs exhibited heterogeneous CD127 expression. We thus determined pSTAT5 signaling on CD127hi Tregs (top 10% of Treg gate) and Tregs exhibiting low but significant CD127 expression (lowest 50% of Treg gate). Following treatment with low doses of IL-7 (1 ng/ml), an approximately 8-fold increase in phospho-STAT5 MFI was detectable among CD127hi Tregs compared to a 2-fold increase in CD127lo cells (Fig. 6B). A similar pattern of STAT5 phosphorylation in the two Tregs sub-populations was observed after treatment with lower or higher doses of IL-7. These results indicate that CD127hi Tregs transduced IL-7 signals more efficiently than CD127lo Tregs.

Because Tregs localized in the bone marrow (BM Tregs) expressed high levels of CD127 at the steady state, we wanted to assess whether BM Tregs could be more reactive to IL-7 than Tregs from LN and spleen. Interestingly, *in vitro* stimulation with IL-7 induced comparable amounts of phospho-STAT5 in conventional and Tregs from BM at all doses studied. Maximal levels of STAT5 phosphorylation were reached in BM Tregs after exposure to very low doses (0.1ng/ml) of IL-7 (Fig 6C). Accordingly, we found that BM Tregs expressed significantly higher levels of phospho-STAT5 than secondary lymphoid organs-derived Tregs upon IL-7 stimulation at all IL-7 doses studied (Fig. 6D).

CD127 is also part of the receptor for thymic stromal-derived lymphopoietin (TSLP). We thus assessed whether differential levels of CD127 expression could influence the capacity of Tregs to react to TSLP as well. TSLP induced a dose dependent increase in STAT5 phosphorylation in both conventional and, to a lesser extent, Tregs (Fig. Supp 1A). No difference in STAT5 phosphorylation was detected in CD127hi and CD127lo Tregs upon TSLP treatment at the doses of 1 and 10 ng/ml and only a slight increase in pSTAT5 was detected in CD127hi Tregs compared to CD127lo Tregs upon exposure to high doses (100ng/ml) of TSLP (Fig. Supp 1B). Similarly to what we performed for IL-7, we assessed whether BM Tregs were more reactive to TSLP than Tregs from LN and spleen. Only a slight increase in pSTAT5 was detected in both conventional and Tregs from the BM upon high doses TSLP treatment (Fig. Supp 1C). Moreover, no significant difference between STAT5 phosphorylation in BM Tregs and Treg from secondary lymphoid organs was observed (Fig. Supp 1C). Collectively, our results demonstrate that Tregs that express more cell surface CD127 are more efficient at perceiving and transducing signals specifically mediated by IL-7.

IL-7 preferentially increases the *in vitro* survival of Tregs expressing high levels of CD127

As we demonstrated that CD127hi Tregs transduced IL-7 signals more efficiently, we assessed whether IL-7 could exert functional effect preferentially on these cells. Conflicting data have been reported so far concerning the role of IL-7 on Treg survival. We thus re-address this question considering high and low CD127 expression on Tregs. To this aim we cultured FACS-sorted total, CD127lo or CD127hi CD4⁺ CD25⁺ cells (Fig. 7A) in the presence or absence of IL-7. Upon overnight incubation, IL-7 significantly increased *in vitro* survival of total CD4⁺ CD25⁺ cells (p=0.003) (Fig. 7B). When considering CD127 expression, a minor but statistically significant increase in cell survival was observed on CD127lo Treg upon IL-7 treatment (p=0.024). Interestingly, a highly significant increase in survival was detected on IL-7 treated CD127hi Treg compared both to non treated cells (p=0.0002) and to IL-7 treated CD127lo cells (p=0.0003). In conclusion, our results indicate that IL-7 preferentially promotes *in vitro* survival of Tregs expressing higher levels of CD127.

DISCUSSION

IL-7 and Tregs are two main regulators of homeostasis and immune responses. In contrast to their constitutive expression of IL-2R α (CD25), Foxp3⁺ CD25⁺ CD4⁺ regulatory T cells have been shown to exhibit low expression of CD127 compared to conventional CD4 and CD8 peripheral T cells [10 –12]. Importantly, regulation of CD127 expression has proven crucial during thymocyte maturation [31] and it has been suggested to be a crucial step for effector or memory differentiation [32 , 33 , 34]. We thus further investigated CD127 expression on Tregs. When dissecting Tregs heterogeneity in lymphoid organs, CD127 expression on Tregs was associated to the expression of CD103 and ICOS. These two markers have been shown to identify activated Tregs [23 , 35]. Using various context of activation (*in vitro* , model of adoptive transfer, skin inflammation model), we demonstrated that high CD127 expression on Tregs was predominant during ongoing immune responses. These data differ from what has been described for conventional T cells which down-regulate transiently IL-7R α expression during activation and suggest different regulation of CD127 expression on conventional and regulatory T cells. Whether TCR ligation and IL-2 signaling differently regulate CD127 expression or whether TCR ligation differs between conventional and regulatory T cells remain to be further investigated. Intrinsic difference in Tregs intracellular machinery may also be responsible for such differential regulation. Interestingly, the opposite regulation of CD127 on Tregs and effector cells during activation led to equivalent CD127 expression on these two subsets when activated. These data provide an additional mechanism for the functional impact of IL-7 on Treg suppression which has been shown to rescue effector cells from Treg mediated apoptosis [4 , 36]. Our data support the hypothesis that Treg and conventional T cells may compete for IL-7 during activation. They also support our previous results demonstrating that IL-7R expression on Tregs was essential for Treg mediated suppression of conventional T cells during lymphopenia-induced proliferation [37].

We also extended our analysis to peripheral tissues that are important localization for activated Tregs. Depending on the organs studied, we observed strikingly different CD127 expression profile on Tregs compared to Tregs isolated from LN. Peripheral blood and secondary lymphoid organs analysis revealed low CD127 expression on Tregs *ex vivo*. Contrasting profiles were observed in mucosal sites: high CD127 expression on Tregs was predominant in the skin but barely detectable in the intestine. Such heterogeneity among various mucosal sites suggests high CD127 expression is not a ubiquitous marker of activated Tregs. Difference in CD127 expression depending on the tissue considered may rely on various mechanisms: differential antigenic load, differential kinetics of activation, differential activation/differentiation pathways and/or specific signals provided in specific sites, differential migration capacities of high CD127 expressing Tregs, differential persistence depending on IL-7 (or TSLP) production on sites [38]. These latter hypotheses may also stand for the high percentage of CD127 expressing Tregs in the BM. The physiological relevance of such specific profile in the BM is currently under investigation and may reflect local IL-7 production. Our data questioned Tregs analyses excluding CD127 expressing cells [39, 40]. Such protocol, commonly used in clinical analyses, allows isolating a highly enriched Treg population but it may also exclude an activated population that could be especially relevant in chronic immune responses and pathological contexts. Although CD127^{hi} Tregs are minor in PBMC, such protocol may prove debatable depending on the tissue and the context considered.

Demonstrating differential CD127 expression among Treg subsets did not formally demonstrate any preferential reactivity to IL-7. To ascertain whether high CD127 expression enhanced IL-7 mediated signaling among Tregs, we determined STAT5 phosphorylation upon *in vitro* IL-7 or TSLP incubation. Two strategies were considered: comparing high and low CD127 expressing Tregs isolated from LN and spleen or total Tregs isolated from secondary lymphoid organs and BM, which exhibit significant difference in CD127 expression. In both analyses, CD127^{hi} Tregs exhibited higher phosphorylation of STAT5. Interestingly, IL-7-induced STAT5 phosphorylation in CD127^{hi} Tregs was comparable with that detected in conventional Foxp3⁻ CD4 T cells. These results confirmed the functional relevance of differential CD127 expression on Tregs in terms of reactivity to IL-7. Similar approach was performed to study the reactivity to TSLP, an IL-7-like cytokine expressed by epithelial cells, including keratinocytes, and important in allergic inflammation [41, 42]. We showed that TSLP induced STAT5 phosphorylation in Tregs but we failed to reveal any association between CD127 expression on Tregs and TSLP signal transduction. Nevertheless, one may hypothesize that activated Tregs could compete with effector T cells for TSLP and this may be one of the mechanisms by which Tregs dampen inflammation at tissue sites. Collectively, these data demonstrated that activated Tregs are an important target of IL-7/IL-7R α signaling. One obvious hypothesis is to consider IL-7 as an additional survival factor for activated Tregs. So far, minor influence of IL-7/IL-7R α signaling on peripheral Tregs has been reported in accordance with the low level of CD127 expression described on Tregs [13–15]. However, Pandiyan et al demonstrated that IL-7 addition enhances Treg survival during *in vitro* activation [43]. High CD127 expression on activated Tregs reconciles these conflicting data since experimental protocols studying Treg sensitivity to IL-7 were performed either on resting [13] or activated Tregs [43]. Indeed, we demonstrated enhanced *in vitro* survival of CD127^{hi} Tregs compared to CD127^{lo} Tregs upon 20h IL-7 incubation. Finally, demonstrating high CD127 expression on activated Tregs may be especially important during lymphopenic episodes [44] and in regard to the development of IL-7 based therapies in cancer and HIV infected patients notably [45–50].

In conclusion, we demonstrate that Tregs exhibit high CD127 expression when activated in contrast to their low expression in non immunized settings. Associated to the recent observation that DCs express CD127 [27], it is remarkable to identify the three main partners involved in immune T cell responses, i.e. conventional T cells, DCs and Tregs, as potential targets of IL-7. Altogether, these data provide additional insights on the ubiquitous impact of IL-7 on various peripheral T cell subsets. Importantly, our data substantiate a direct link between two main components of immune regulation that are Tregs and IL-7 and identify activated Tregs as potential target of endogenous or therapeutic IL-7.

MATERIALS AND METHODS

Mice

6 to 8 weeks old C57Bl/6 expressing CD45.1 or CD45.2 (Charles River, Janvier) and B6 RAG-2 deficient mice were used as donors and hosts respectively. Foxp3-GFP mice were purchased from the Jackson Laboratory. All mice were kept under specific pathogen free conditions and all experiments were performed according to institutional guidelines of the European Community.

Cell suspension

Single cell suspensions were prepared from LN, SP, liver, thymus and BM in HBSS containing 2% FCS (both from PAA Laboratories GmbH). For preparation of intestinal cell suspensions, colon pieces were incubated in PBS containing 5 mM EDTA for 15 min on a shaking incubator at 37°C prior digestion. Supernatant was collected and remaining tissue was further digested. To digest tissues, intestine, lung and ear skin were finely minced and stirred in HBSS with 400 μ g/ml Liberase (Roche) and 50 μ g/ml Collagenase IV (Sigma) for 1 hour at 37°C. Cells isolated from intestine, liver and lung were pelleted, resuspended in 37% Percoll (GE Healthcare) and spun at 2,500 rpm for 25 min. Isolated lymphocytes were washed twice and used for subsequent flow cytometry analyses.

Flow cytometry

Extracellular staining was preceded by incubation with purified anti-CD16/32 antibodies (FcγRII/III block, 2.4G2) (eBioscience) to block nonspecific staining. Cells were stained with FITC-, PE-, PEcy5-, PEcy7- APC- and APCAlexa750-labeled or biotinylated appropriate antibodies including CD4 (GK1.5); TCR (H57-597); CD62L (MEL-14); CD69 (H1.2F3); CD25 (PC61.5); CD127 (A7R34); ICOS (7E.17G9); CD103 (2E7) or appropriate isotype Abs. Streptavidin-FITC, PEcy5 or PEcy7 were used to develop biotinylated Abs. All Abs were purchased from eBioscience. Intracellular Foxp3 staining was performed using eBioscience APC-conjugated Foxp3 staining buffer set (FJK-16s). Six-color flow cytometry was performed with a FACSCanto cytometer (BD Biosciences) and data files were analyzed using FlowJo software (Tree Star Inc).

Cell purification

Cell sorting of naive CD4 T cells (CD4⁺ CD62L⁺ CD25⁻) and Tregs (CD4⁺ CD25⁺) was performed on a FACSVintage cell sorter (BD Biosciences). For suppressive assays and adoptive transfer experiments, Tregs subsets were sorted based on CD62L, CD69, ICOS, CD103 expression following positive selection using anti-CD25 PE or biotinylated antibodies incubation, and purification with anti-PE or anti-biotin beads respectively (Miltenyi). For in vitro survival experiments, cells were isolated from mesenteric lymph nodes. CD127hi Tregs (top 10% of Treg gate) and Tregs exhibiting low CD127 expression (approximately lowest 50% of Treg gate) were next FACS sorted among CD4⁺ CD25⁺ cells.

In vitro cell cultures

Cells were cultured in RPMI 1640 medium containing penicillin, streptomycin, L-glutamine, HEPES buffer, non-essential amino acids, sodium pyruvate and beta-mercaptoethanol and 10% heat inactivated FCS (all from PAA Laboratories GmbH). For activation experiments, total CD4 T cells (1×10^5) were cultured in 96-well plates with splenocytes from RAG^{-/-} mice (1×10^5) and 0.5µg/ml anti-CD3.

Suppression assays

Suppression assays were performed using allotypic marker CD45.1/CD45.2 to distinguish conventional from CD25⁺ sorted population. CFSE (Sigma) labeled CD45.1⁺ CD62L⁺ CD25⁻ CD4⁺ cells (3×10^4) were cultured for 72h-96h in 96-well plates with splenocytes from RAG^{-/-} mice (3×10^4), 0.5µg/ml anti-CD3 (eBiosciences), in presence or absence of the indicated subset of CD4⁺ CD25⁺ CD45.2⁺ cells. CFSE labeling was performed using standard methods.

Adoptive transfer experiments

FACS sorted CD45.1 naive CD4 T cells (1×10^5) and CD45.2 regulatory T cells (0.5×10^5) were co-injected into RAG2-deficient mice. Six weeks after transfer, spleen and mesenteric LN were harvested.

Contact dermatitis model

Acute skin inflammation experiments were conducted as described [28]. Briefly, 0.5% 2,4-dinitrofluorobenzene (DNFB) (Sigma) diluted in acetone/olive oil vehicle (4:1 vol/vol) was applied onto ears of C57BL/6 mice. Control mice were treated with vehicle alone. Six days later, ear thickness was measured with a digital caliper, cervical draining lymph nodes and ears were isolated.

STAT5 phosphorylation experiments

Cells isolated from Foxp3-GFP mice were incubated for 30 min at 37 °C with or without IL-2, IL-7 (Immuno Tools) or TSLP (R&D) and immediately fixed in 2% paraformaldehyde. Cells were made permeable by incubation in 90% methanol and then were stained with primary rabbit antibody to phosphorylated STAT5 or isotype control (Cell Signaling) revealed with an anti-rabbit Alexa-647 conjugated secondary antibody.

In vitro survival experiments

5×10^4 FACS-sorted total CD4⁺CD25⁺, CD4⁺CD25⁺CD127lo or CD4⁺ CD25⁺CD127hi were cultured overnight (18–20h) in medium alone or in the presence of IL-7 (10 ng/ml). Cell survival was determined using the fluorescence-based LIVE/DEAD assay (Invitrogen).

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List of abbreviations

ICOS : Inducible Costimulatory molecule

DNFB : 2,4-dinitrofluorobenzene

SP : Spleen

PB : Peripheral Blood

PC : Peritoneal Cavity

TSLP : thymic stromal-derived lymphopoietin

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FIGURE 1

In lymph nodes, CD103 and ICOS expression identifies Tregs presenting high levels of CD127

(A) Phenotypic heterogeneity of Tregs. Dot plots show the expression of CD62L, CD69, CD103 and ICOS depending on Foxp3⁺ expression on CD4⁺ TCRβ⁺ LN cells. Percentages of cells from a representative sample are indicated in each quadrant. (B) Suppression assay of the indicated CD4⁺ CD25⁺ subpopulations. CFSE profiles of sorted naïve CD4 T cells cultured 72–96 h in the presence of 0.5 µg/ml anti-CD3 with or without sorted CD4⁺ CD25⁺ subsets (filled and open profile respectively). Data are representative of three independent experiments. (C) CD127 expression on total and CD62L, CD69, CD103 or ICOS expressing (grey histogram) or non expressing (thick open line) Foxp3⁺ Tregs compared to conventional Foxp3⁻ CD4 T cells (open histogram). Isotype control staining for both Treg subsets are represented (dotted and shaded lines). (D) Dot plot shows CD103 and ICOS expression on LN CD4⁺ Foxp3⁺ Tregs. Histograms show CD127 expression on total Tregs (thick open line) or indicated Treg subsets identified based on ICOS and/or CD103 expression (gray shaded profile) compared to conventional CD4 T cells (open histogram). Isotype of total Tregs and Treg subsets are presented (dotted and dashed lines respectively). FACS data are representative of more than 5 independent experiments, using at least three C57Bl/6 mice per experiment.

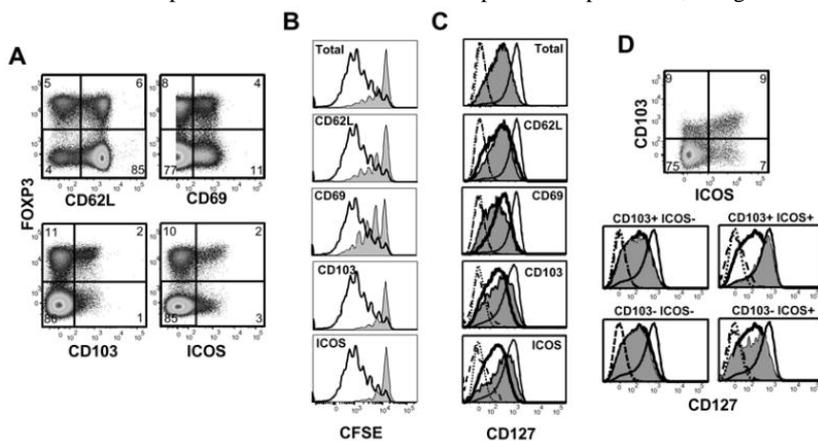


FIGURE 2

Tregs up-regulate CD127 upon in vitro activation

Total CD4⁺ cells isolated from LN and SP of three to five C57Bl/6 mice were activated in vitro with anti-CD3 for 4 days. (A) Dot plots represent ICOS and Foxp3 expression on fresh (left plot) and stimulated CD4 T cells (right plot). (B) CD127 expression on conventional Foxp3⁻ (upper panel) and regulatory Foxp3⁺ (lower panel) CD4⁺ cells upon in vitro activation. CD127 expression on control cells (open profile) or on activated cells (gray shaded profile) is shown. Isotype controls staining of both activated and ex vivo population are represented by the dotted and dashed lines respectively. (C) CD127 MFI are the mean \pm SEM of three independent experiments. The p-values were determined using 2 tailed Mann Whitney U-test (* p<0.05)

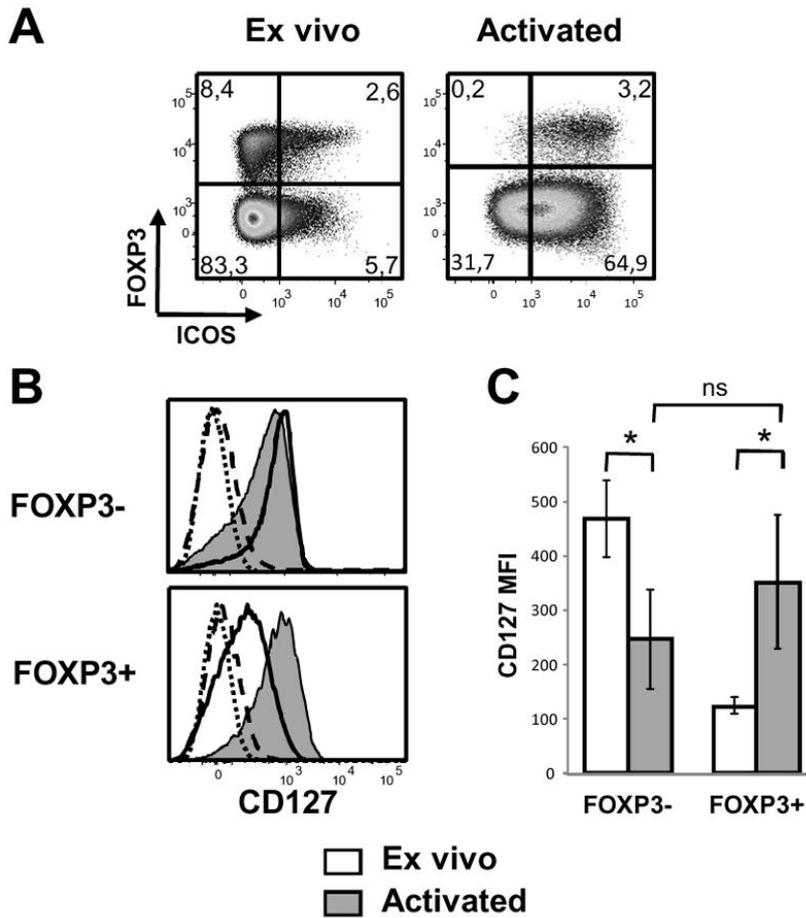


FIGURE 3

Tregs up-regulate CD127 upon in vivo activation

FACS sorted CD45.1⁺ naïve CD4 T cells and CD45.2⁺ Tregs or “naïve-like” Tregs were co-injected into RAG-deficient mice. Phenotypic analysis was performed on mesenteric LN and spleen 6 weeks after transfer. (A, C) Histograms showing CD127 expression on conventional Foxp3⁻ (upper panel) and regulatory Foxp3⁺ (lower panel) CD4⁺ cells during in vivo activation when total (A) or “naïve-like” Tregs (C) were co-injected. Isotype controls of activated and control fresh cells are represented by dotted and dashed lines. (B, D) Graphs representing CD127 MFI on CD4 T cells isolated from control WT mice (open profile) or transferred cells (gray shaded profile) when total (B) or “naïve-like” Tregs (D) were co-injected. Data are the mean ± SEM of three independent experiments using two to three mice per group in each experiment. The p-values were determined using 2 tailed Mann Whitney U-test (* p<0.05, **p<0.01).

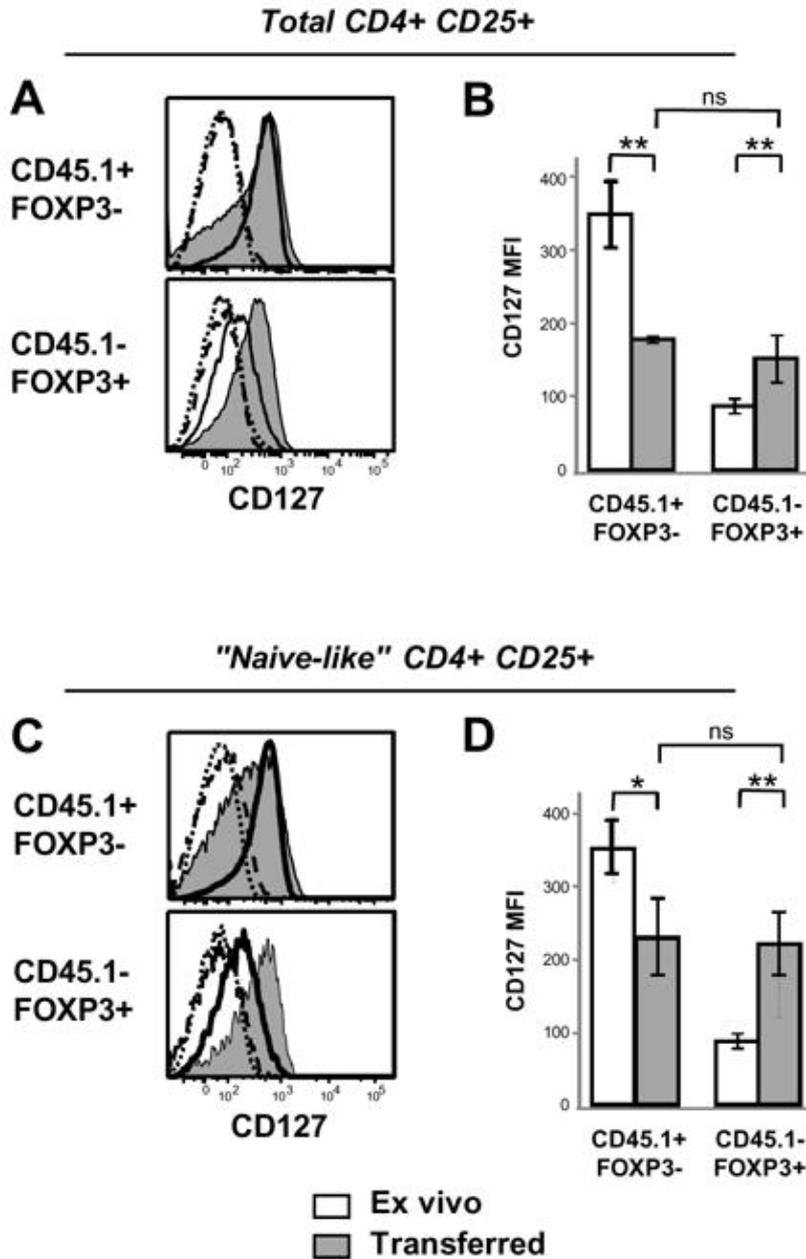


FIGURE 4

High CD127 expression on activated Tregs in a model of acute skin inflammation

(A) Ear thickness and (B) CD4 T cell numbers in cervical lymph nodes draining ear skin 6 days after DNFB treatment. Data are the mean \pm SD of five mice from a single experiment representative of at least three independent experiments. The p-values were determined using 2 tailed Mann Whitney U-test (** $p < 0.01$). Dot plots in (C) and (D) represent ICOS and CD127 expression on CD4⁺ Foxp3⁻ and CD4⁺ Foxp3⁺ T cells from, respectively draining LN and ear skin. Histogram profiles show CD127 expression on ICOS⁻ (thick black line) or ICOS⁺ (gray shaded profile) cells from DNFB treated mice. Isotype controls of ICOS⁻ and ICOS⁺ subsets are represented by the dotted and dashed lines respectively. This figure is representative of at least three independent experiments using pool of five to fifteen mice per experiment.

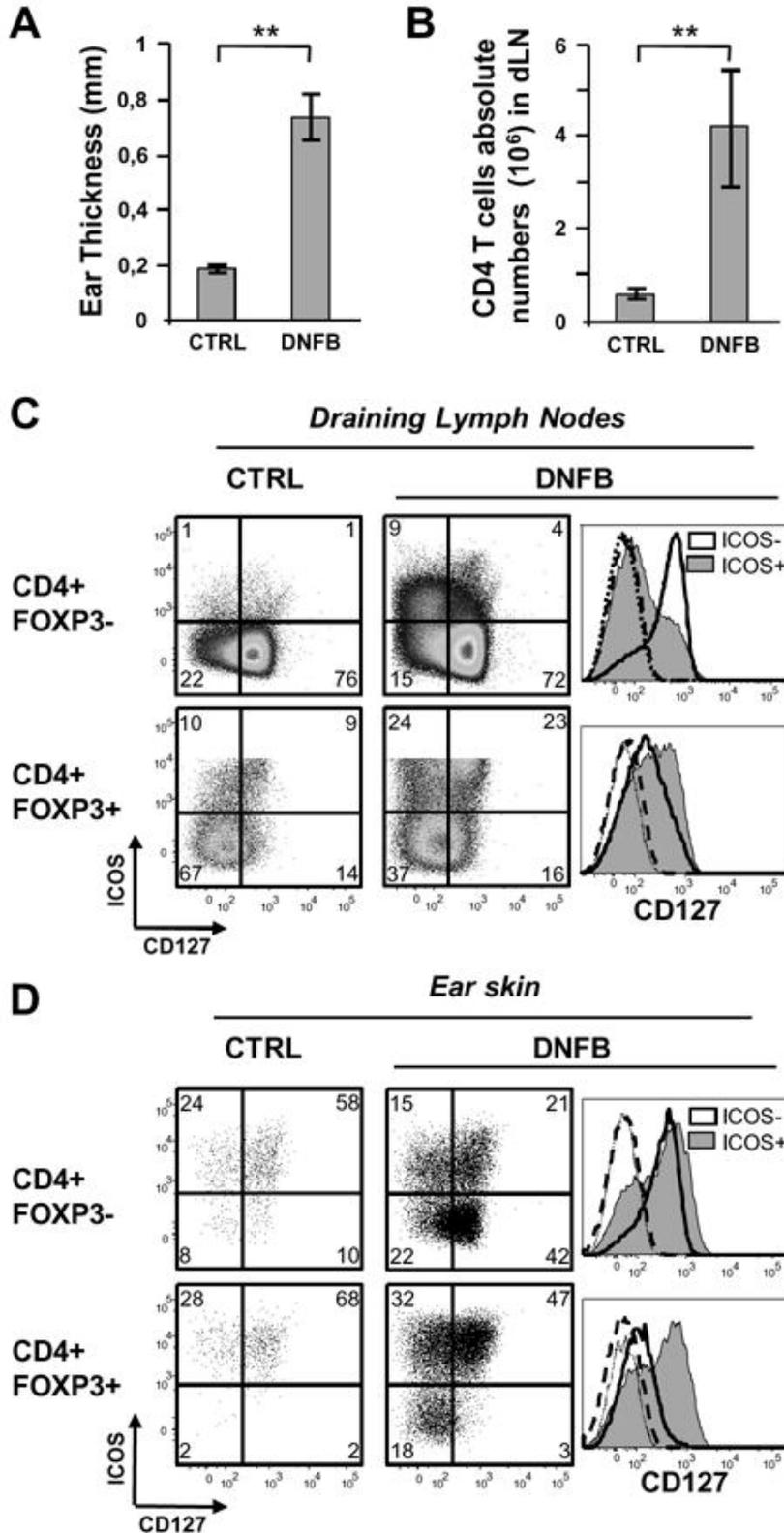
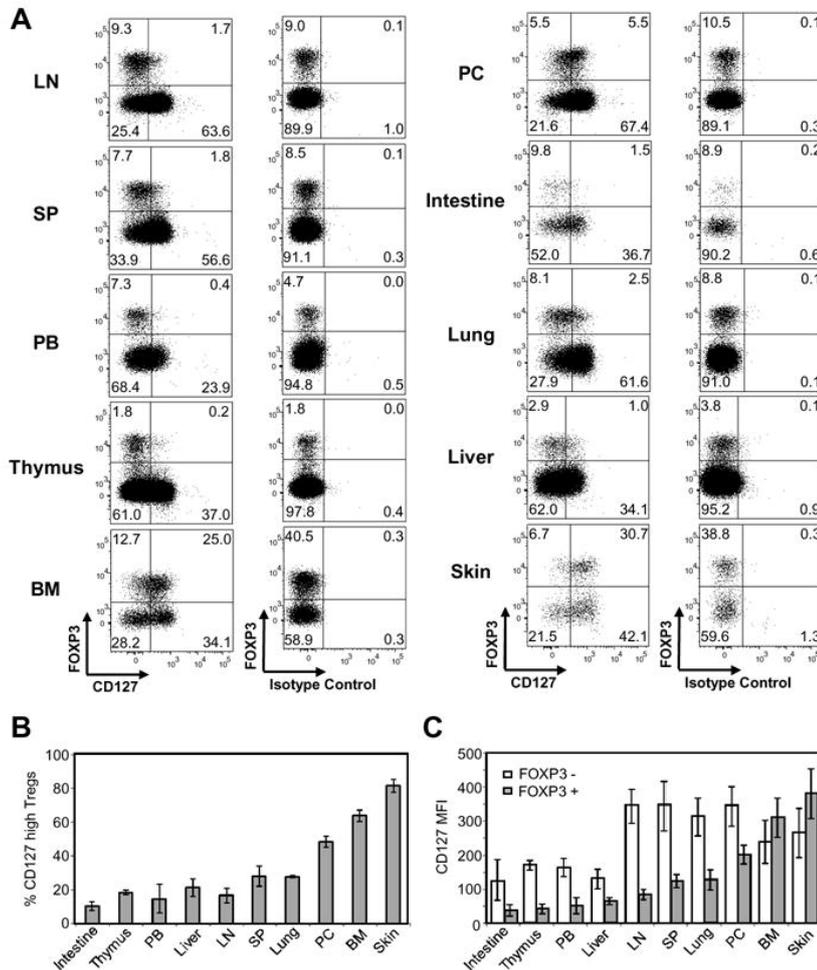


FIGURE 5

Different CD127 expression on Tregs depending on the organs studied

(A) Dot plots represent ex vivo expression of CD127 or isotype and Foxp3 on CD4⁺ TCRβ⁺ cells isolated from LN, SP, peripheral blood (PB), thymus, BM, peritoneal cavity (PC), intestine, lung, liver and skin of C57Bl/6 mice. Percentages of cells in each quadrant are indicated. Data are representative of three independent experiments. (B) Percentages of CD127 expressing cells among Foxp3⁺ CD4 T cells in different organs are shown. Data are the mean ± SEM of at least three independent experiments. (C) CD127 MFI on Foxp3⁻ (white bars) and Foxp3⁺ (grey bars) CD4 T cells are shown. Error bars represent SEM of three independent experiments. Data are representative of at least three independent experiments, with five to fifteen mice pooled in each experiment depending on the organs studied.

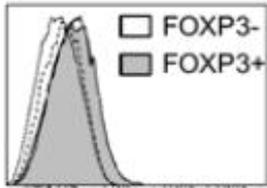
**FIGURE 6**

Different IL-7 signal transduction in Tregs depending on CD127 expression

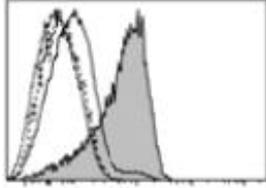
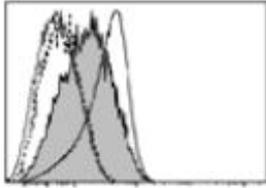
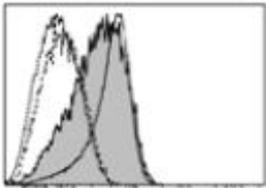
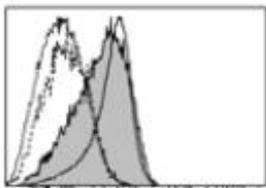
Cells from Foxp3-GFP were incubated with either medium alone, IL-2 (50 U/ml) or IL-7 (0.1, 1, 10ng/ml) for 30 min. STAT5 phosphorylation was determined on Foxp3⁺ or Foxp3⁻ cells isolated from pooled LN and splenic cells (A) or BM cells (C). (B) p-STAT5 staining depending on CD127 expression among Foxp3⁺ LN and SP cells, CD127^{hi} Tregs were identified by gating on top 10% CD127 expressing CD4⁺ GFP⁺ cells (grey shaded profile); CD127^{lo} Tregs were defined as 50% of CD4⁺ GFP⁺ cells expressing the lowest CD127 levels (open profile). (D) Comparison of p-STAT5 staining among LN and SP or BM isolated Tregs. Results are representative of three independent experiments, using one GFP Foxp3 mouse for each experiment.

A

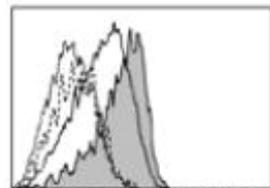
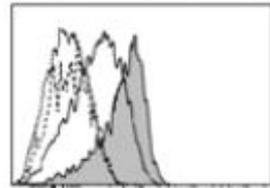
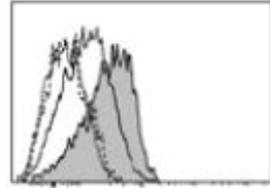
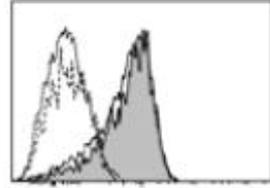
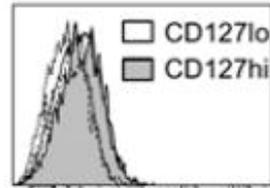
NT



IL-2

IL-7
0.1IL-7
1IL-7
10

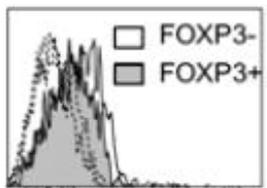
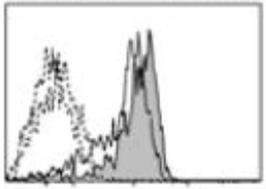
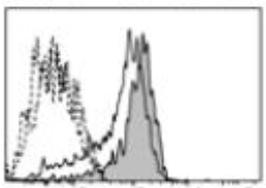
pSTAT5

B

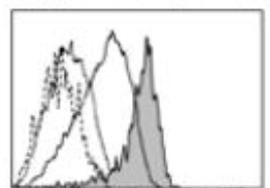
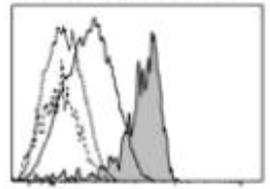
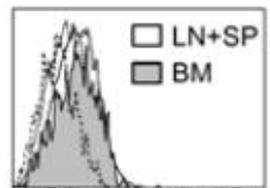
pSTAT5

C

NT

IL-7
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pSTAT5

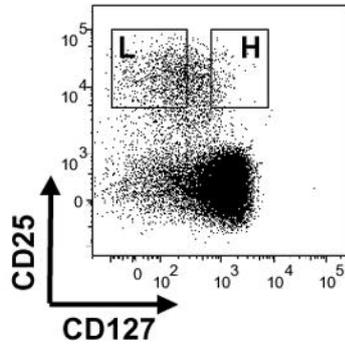
D

pSTAT5

FIGURE 7

IL-7 preferentially promotes in vitro survival of Tregs expressing higher levels of CD127

FACS-sorted total, CD127^{lo} or CD127^{hi} CD4⁺ CD25⁺ cells were cultured overnight in medium alone or in the presence of IL-7 (10 ng/ml). (A) Gating strategy to isolate the top 10% CD127 expressing CD25⁺ T cells (H gate) and CD127^{lo} expressing Tregs (L gate) by FACS cell sorting. (B) Percentage of live cells after culture of indicated Treg populations in medium alone (open bars) or in the presence of IL-7 (filled bars). FACS sorting was performed on pooled mesenteric LN isolated from fifteen mice for each experiment. Data are the mean \pm SD of triplicates from a single experiment representative of three. The p-values were determined using unpaired 2 tailed Student t-test (*p<0.05, **p<0.01, ***p<0.001).

A**B**