



Research paper

Expanded murine regulatory T cells: Analysis of phenotype and function in contact hypersensitivity reactions

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Abstract

Regulatory T cells (Treg) exert suppressive functions in the periphery of the body for the maintenance of tolerance. The functional analysis of Treg is hampered by the fact that only small numbers (5%–10% among the CD4⁺ T cells) of Treg exist in peripheral blood and tedious isolation methods further reduce the yield of high-purity Treg.

We therefore set out to expand isolated murine Treg in ex vivo cultures with help of anti-CD3/anti-CD28 antibodies in the presence of IL-2. Our expansion-protocol described herein resulted in a 200-fold expansion of Treg and does not involve feeder cells, beads or other cellular compounds that would interfere with further in vivo use of the expanded cells. Expanded Treg could even be stored in liquid nitrogen and thawed without loss of function. Functional analysis revealed that expanded Treg are superior suppressors of T cell functions in vitro and in vivo and when applied in a model for contact hypersensitivity reactions, Treg were able to suppress the ear swelling reaction significantly. Thereby the expanded Treg home to secondary lymphoid organs in similar manner as observed for freshly isolated Treg. Accordingly, the expansion procedure does not effect the expression of specific homing markers.

Thus, this protocol will facilitate the production of Treg as an “off-the-shelf reagent” and offers the possibility to explore the application of Treg as a cellular therapeutic in several allergic and autoimmune diseases.

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1. Introduction

Naturally occurring CD4⁺CD25⁺ regulatory T cells (Treg) have been characterized by their essential role in

the maintenance of peripheral tolerance. Their regulatory function has been evidenced in several animal models of autoimmune diseases (Sakaguchi et al., 1995) as well as in tumors (Onizuka et al., 1999) and graft versus host disease (Taylor et al., 2001). Likewise, in humans the absence of Treg has been associated with the appearance and the course of severe autoimmunity, as shown by the X-linked inheritance syndrome (IPEX) or by the onset of multiple sclerosis (Bacchetta et al., 2006; Viglietta et al., 2004). Further studies underline the crucial role of Treg in preventing transplant rejection or

Abbreviations: Treg, regulatory T cells; CHS, contact hypersensitivity; TCR, T cell receptor.

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graft versus host disease, demonstrating the wide range of potential therapeutically use of Treg.

Originating in the thymus, Treg represent only 5–10% of the mature CD4⁺ T cell pool in mice and less than 5% in humans, which hampers the functional analysis and the clinical application of these regulatory cells. In order to considerably increase the number of Treg, different methods of polyclonal or antigen-specific *in vitro* expansion of both murine and human CD4⁺CD25⁺ regulatory T cells have been developed so far. Since Treg are anergic to TCR-mediated stimulation, obtaining sufficient numbers of pure CD4⁺CD25⁺ T cells for therapeutic use requires protocols, which are able to break the anergic state of Treg. For the effective polyclonal proliferation of murine as well as human Treg the repeated stimulation via TCRs and CD28 in combination with high amounts of recombinant IL-2 is described. Thus, using anti-CD3/CD28-coated microbeads the number of Treg increased up to 225-fold within 11 days in case of murine cells (Tang et al., 2004) and up to 40,000-fold in the case of highly purified human CD4⁺CD25^{high} T cells (Hoffmann et al., 2004). More limited numbers of polyclonal or antigen-specific expanded Treg were generated with IL-2 together with autologous DCs, allogeneic stimulation, or with antigen-loaded antigen-presenting cells (APCs) (Tarbell et al., 2004; Cohen et al., 2002).

Expanded Treg, irrespective of the protocol used, retained the characteristic cell surface phenotype, including high expression of CD25, CD62L, Foxp3, CTLA-4 and GITR. Functional analysis of the expanded cells showed that the suppressive function *in vitro* as well as *in vivo* was not diminished by the procedure of culture; some studies even describe an increase in suppressive activity compared with freshly isolated Treg (Yamazaki et al., 2003; Hoffmann et al., 2004; Tang et al., 2004). Thereby, the mechanisms of regulation are still unclear and the necessity of cell–cell contact *in vitro* versus the requirement of soluble, immunosuppressive factors, like TGF- β or IL-10 *in vivo*, is still discussed.

In the present study, we established a protocol for the *in vitro* expansion of murine CD4⁺CD25⁺ regulatory T cells achieving an up to 180-fold enhancement of the cell numbers within 23 days. Thereby we could show that the expanded cells exert enhanced suppressive capacity *in vitro* and *in vivo* in the murine model of contact hypersensitivity (CHS). Moreover, when the expanded cells were frozen and thawed before use, they exerted significant suppressive function in our CHS model.

The increase of function was associated with an augmentation of Foxp3 expression during constant expression of other characteristic surface markers of regulatory T cells. The migration pattern and the cytokine production

of the expanded Treg were unchanged after three weeks of culture, verifying the comparability of freshly isolated and expanded Treg. Thus, since Treg are important to control autoimmunity and are potential targets of immunotherapy, our results show a possibility to enhance the suppressive activity of Treg and to successfully preserve fully functional Treg by freezing.

2. Materials and methods

2.1. Mice

BALB/c mice, originally purchased from Charles River (Sulzfeld, Germany), were bred at the central animal facility of the University of Heidelberg. Animals were used for experiments at the age of 6–12 weeks and within a single experiment mice were sex and age matched. All experiments were approved by the State of Baden Württemberg.

2.2. Reagents and antibodies

CD4⁺CD25⁺ regulatory T Cell Isolation Kit from Miltenyi Biotec (Bergisch Gladbach, Germany); anti-CD3 (145-2C11) and anti-CD28 (37.51), BD Biosciences (Heidelberg, Germany); rm IL-2, R&D Systems (Wiesbaden, Germany); PKH26 red fluorescent cell linker kit, Sigma-Aldrich (St. Louis, MO); TNCB, Sigma-Aldrich (Milwaukee, WI); FITC Foxp3 (clone FJK-16s) staining set from eBioscience, San Diego, Ca, USA; OptEIA™ mouse IL-10 ELISA Set, BD Biosciences, San Diego, Ca, USA; for flow cytometry all mAb were purchased from BD Biosciences.

Cell culture medium (cRPMI) consisted of RPMI 1640 (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 75 mM Hepes (Roth GmbH, Karlsruhe, Germany), 100 U/ml penicillin, 100 g/ml streptomycin and 2 mM L-glutamine (Invitrogen GmbH, Karlsruhe, Germany), and 10% heat-inactivated FCS (PAA).

2.3. Purification of T cell subsets

CD4⁺ T cell subpopulations were isolated from spleen and/or lymph nodes using magnetic bead based separation technique. Single cell suspensions were incubated with biotin-conjugated Ab against CD8a, CD11b, CD45R, DX5 (rat IgM) and Ter-119 (rat IgG2b) followed by anti-biotin MicroBeads. CD4⁺ cells were negatively selected on midi-MACS columns. Purity averaged out > 97%. CD4⁺ T cells were incubated with anti-CD25 PE mAb and staining with anti-PE MicroBeads. The CD4⁺CD25⁺ T cell subpopulation

was positively selected according to the manufacturers instructions. The purity of CD4⁺CD25⁺ T cells averaged 90–95%. Conventional CD4⁺CD25[−] T cells were concentrated in the flow-through.

The regulatory function of the CD4⁺CD25⁺ T cells was always proved in proliferation assays in co-culture with CD4⁺CD25[−] T cells stimulated with anti-CD3 and anti-CD28.

2.4. Expansion of CD25⁺ regulatory T cells

CD25⁺ regulatory T cells isolated from LN or spleen or pooled cells were cultured in a 96 well round-bottom plate in a concentration of 1×10^6 /ml. Plates were coated over night with anti-CD3 (10 µg/ml) before adding the cells, complete medium was enriched with anti-CD28 (0.5 µg/ml) and 1000U/ml rm IL-2. Depending on the condition of the cells and the proliferation rate, complete medium+rm IL-2 was added to the culture or cells were split and transferred to 24 well or 6 well plates.

Freezing of the expanded CD25⁺ T cells was generally carried out on day 12 of culture in complete medium/FCS/DMSO (1/2/0.2).

2.5. In vitro functional analyzing of regulatory T cells

Freshly isolated CD4⁺CD25[−] T cells from BALB/c mice were cultured in 96 well round-bottom plates (1×10^6 cells/ml). Stimulation was carried out with anti-CD3 and anti-CD28 (0.5 µg/ml each). Fresh isolated, expanded or thawed CD25⁺ T cells were added in different concentrations. Cell proliferation was determined by measuring thymidine incorporation after 4 days of culture and the addition of 0.5 µCi/ml [³H]-thymidine (Amersham, Freiburg, Germany) for the last 18 h. Cells were harvested and thymidine incorporation was determined using a PerkinElmer scintillation counter. The same procedure was also carried out in transwell experiments.

Secreted cytokines were measured by sandwich enzyme linked immunosorbent assay (ELISA) using paired antibodies. Intracellular expressions of cytokines were analyzed by flow cytometry.

2.6. Contact hypersensitivity (CHS)

For the CHS reaction on mouse ears, mice were sensitized by applying 15 µl of 1% 2,4,6-trinitro-1-chlorobenzene (TNCB) (Sigma-Aldrich) dissolved in acetone/oil (4:1) on the shaved abdomen on day 0. On day 5, ear thickness was measured using a mechanical length measuring instrument (Oditest, Kroeplin, Schlüch-

tern, Germany). Afterwards mice were challenged by application of 10 µl of 0.5% TNCB solution dissolved in acetone and olive oil mixture (4:1) on each site of the right ear. After 24 h the ear thickness was measured again and difference was calculated as Δ ear response in mm $\times 10^{-2}$ (mean $\pm$ SD).

2.7. Fluorescence labeling

Freshly isolated or expanded CD4⁺CD25⁺ Tregs were labeled with the membrane inserting dye PKH26. 2×10^7 serum free cells were resuspended in 1 ml diluent C before adding 1 ml of freshly prepared membrane linkers (4 µM in the corresponding diluent). After 3 min incubation at room temperature, reaction was stopped with 2 ml FCS within 1 min. 4 ml medium was added and labeled cells were collected by centrifugation (400g, 10 min, 25°C). During the staining procedure about 20% of inserted cells were lost. 90–95% of the remaining fluorescence labeled cells was viable as assessed by trypan blue staining. Inserting of dyes did not influence proliferation or cytokine production of these cells, as we examined in in vitro assays.

3. Results

3.1. In vitro expansion of CD4⁺CD25⁺ regulatory T cells from different lymphoid organs

To establish the expansion of Treg in vitro, we cultured freshly isolated CD25⁺ T cells (purity $\geq 96\%$) from spleen and lymph node (LN) in 96 well round-bottom plates, coated with plate-bound anti-CD3 (10 µg/ml). Cell culture was performed in complete RPMI medium supplemented with anti-CD28 (0.5 µg/ml) and IL-2 in high concentration (1000U/ml). In the course of the culture, cells were examined carefully, fresh medium was added and cells were split or transferred into 24 well or 6 well plates respectively, if required. During culture, the medium always contained 1000U/ml IL-2, whereas anti-CD3/CD28 stimulation was not renewed. Analyzing the expansion-pattern of the Treg we measured an increase in the proliferation rate after 5 days of culture, which reached its maximum after approximately 23 days (Fig. 1A). At that time point the number of cells has increased up to 180-fold as compared to the original number of Treg.

We next asked, whether the origin of the cells, i.e., LN or spleen, has an impact on the expansion of the Treg. Therefore, we isolated Treg from the respective organs and analyzed the cell numbers at different time points of the culture. The expansion curves in Fig. 1B

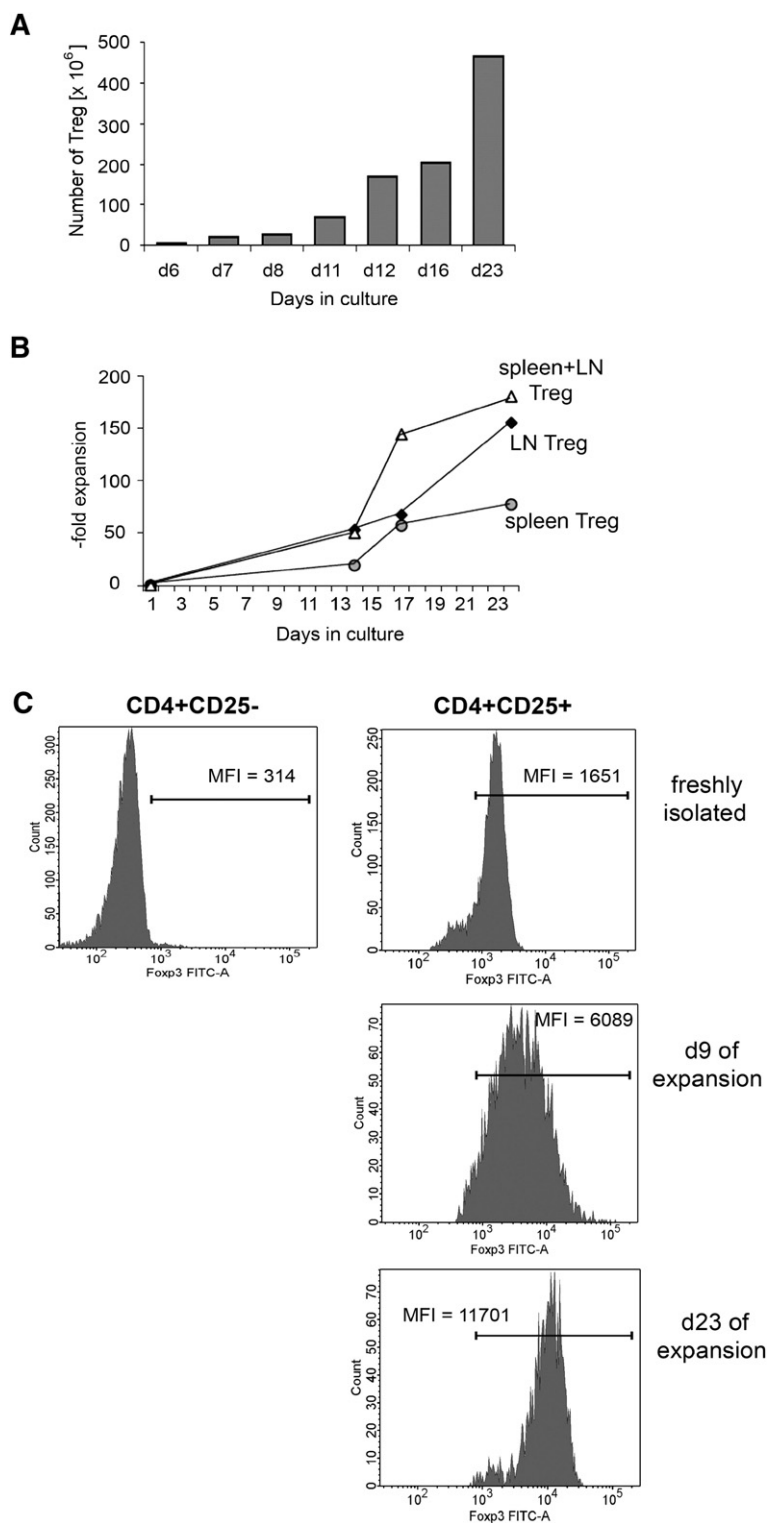


Fig. 1. Expansion of Treg resulted in an increased expression of Foxp3. CD4⁺CD25⁺ regulatory T cells isolated from lymph node (LN) and spleen were expanded with plate-bound anti-CD3, anti-CD28 and recombinant mouse IL-2. (A) The number of Treg increased continuously over 23 days from 3×10^6 to 464×10^6 cells. (B) Thereby the origin of the cells was crucial for the magnitude of expansion, as pooled spleen and LN derived Treg expanded better than separated LN or spleen derived Treg, respectively. (C) FACS analysis of conventional CD4⁺CD25⁻ T cells, freshly isolated Treg and expanded Treg showed that MFI (mean fluorescence intensity) of Foxp3 increased in the course of expansion. Shown are always the results from one experiment out of ten.

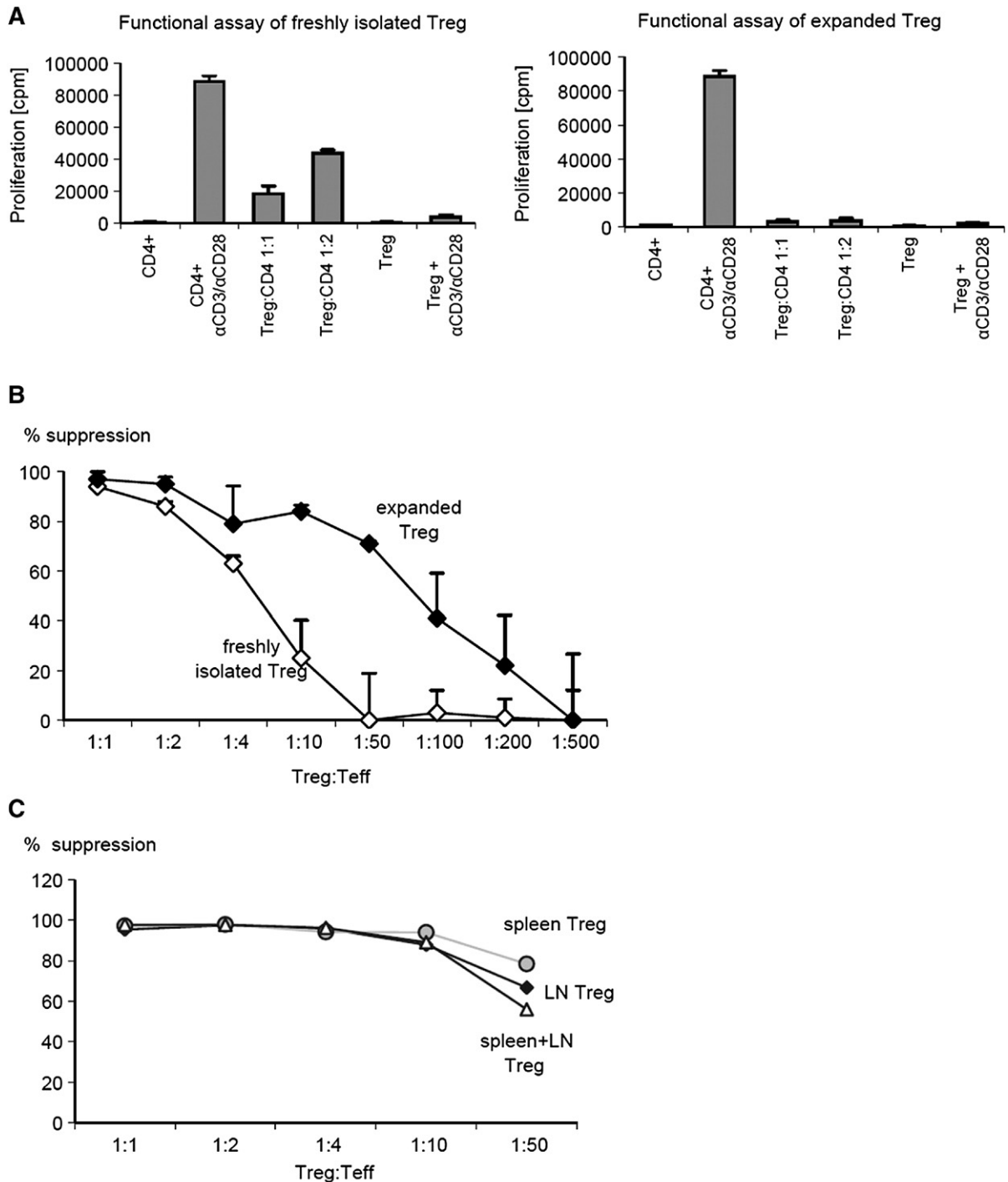


Fig. 2. Expanded Treg display superior suppressive functions in vitro. Conventional CD4⁺CD25⁻ T cells (Teff) were stimulated with anti-CD3/CD28 and cultured in ratios as indicated with freshly isolated Treg (A, left) or expanded Treg (A, right), which were harvested on day 15 of culture. As controls unstimulated CD4⁺ and Treg, as well as stimulated Treg, were cultured in the same plate. Expanded Treg exert an increased suppressive activity (A). Titration of the Treg/Teff ratio demonstrated that expanded Treg still suppress the Teff proliferation, when freshly isolated Treg did not show any inhibiting capacity any more (ratio 1/50) (B). The origin of expanded Treg did only influence their suppressive capacity when used in a 1/50 ratio (C). Data shown represent the mean±SD of five independent experiments (A+B) and one representative experiment out of three (C).

demonstrate that Treg isolated from LN and mixtures of spleen and LN derived Treg gave the highest yields of cells after 23 days of culture (180-fold and 155-fold expansion respectively). In contrast, expanded Treg isolated from spleen proliferated slower and reached only a 77-fold expansion in the same culture period.

During the expansion of Treg, independent of the origin, the percentage of cells expressing the typical surface receptors for regulatory T cells, such as CD25, CD62L, CD103 and Foxp3, did not change (not shown) as compared to freshly isolated Treg. However, we observed increased mean fluorescence intensity (MFI) of Foxp3 during expansion in correlation to the time spent in culture (Fig. 1C).

3.2. Expanded Treg display superior suppressive functions in vitro

To analyze the suppressive function of the expanded Treg in vitro, we co-cultured the cells with conventional CD4⁺ T cells in presence of anti-CD3/anti-CD28. Fig. 2A shows that proliferation of CD4⁺ T cells is substantially more inhibited by expanded Treg as compared to freshly isolated Treg when applied in a 1:1 ratio. When the number of the Treg in the assay was gradually reduced, i.e., when Treg were added to responder T cells in a 1:50 ratio, the freshly isolated Treg did not exert any suppressive function, whereas the expanded Tregs inhibited the proliferation of CD4⁺ cells up to 70% (Fig. 2B). This superior function was independent from the origin of the Treg, as spleen derived as well as LN derived Treg and mixtures thereof, suppressed the proliferation of CD4⁺ T cells equally well (Fig. 2C).

In light of the current literature, suggesting that Foxp3 acts as marker for the suppressive potency of Treg, these results indicate that the expanded and thus Foxp3 “very high” Treg have increased suppressive capacity as compared to freshly isolated controls.

3.3. Expanded Treg exert their suppression in vitro via cell to cell contact

To further address the means by which this increased inhibitory function is conveyed, we analyzed the secretion of the major inhibitory cytokine IL-10 by Treg. Fig. 3A shows that both cell types, expanded as well as fresh Treg, produce comparable amounts of IL-10 upon stimulation. Using intracellular staining of IL-10 followed by FACS analysis (Fig. 3B) we further demonstrate that the basal levels of IL-10 production (expanded Treg compared to fresh Treg) on a per-cell basis are increased in expanded

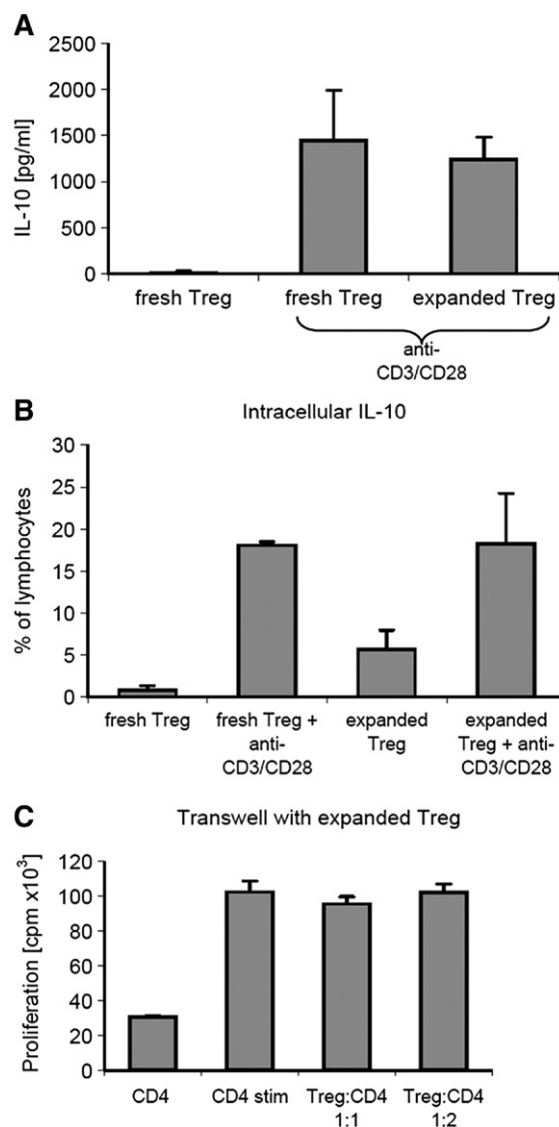


Fig. 3. Expanded Treg exert their suppression in vitro via cell to cell contact. Freshly isolated and expanded Treg were cultured with plate-bound anti-CD3 and anti-CD28 for 48 h. Expanded Treg were washed 3 times in complete medium before stimulation. In both Treg populations comparable IL-10 production was measured in the supernatant using ELISA (A) and in the cells by intracellular FACS analysis (B). Using Boyden-chambers for the functional assays, which are described in detail for Fig. 2A, the suppression, mediated by expanded Treg, was abrogated (C). Data shown represent the mean $\pm$ SD of five independent experiments (A + B) and of three independent experiments in C.

Treg, but upon stimulation both cell types produce similar amounts of IL-10. Moreover, consistent with previous results obtained with freshly isolated regulatory T cells, the observed inhibitory effect was dependent on cell to cell contact, as Boyden-chamber experiments (Fig. 3C) abrogated the suppression.

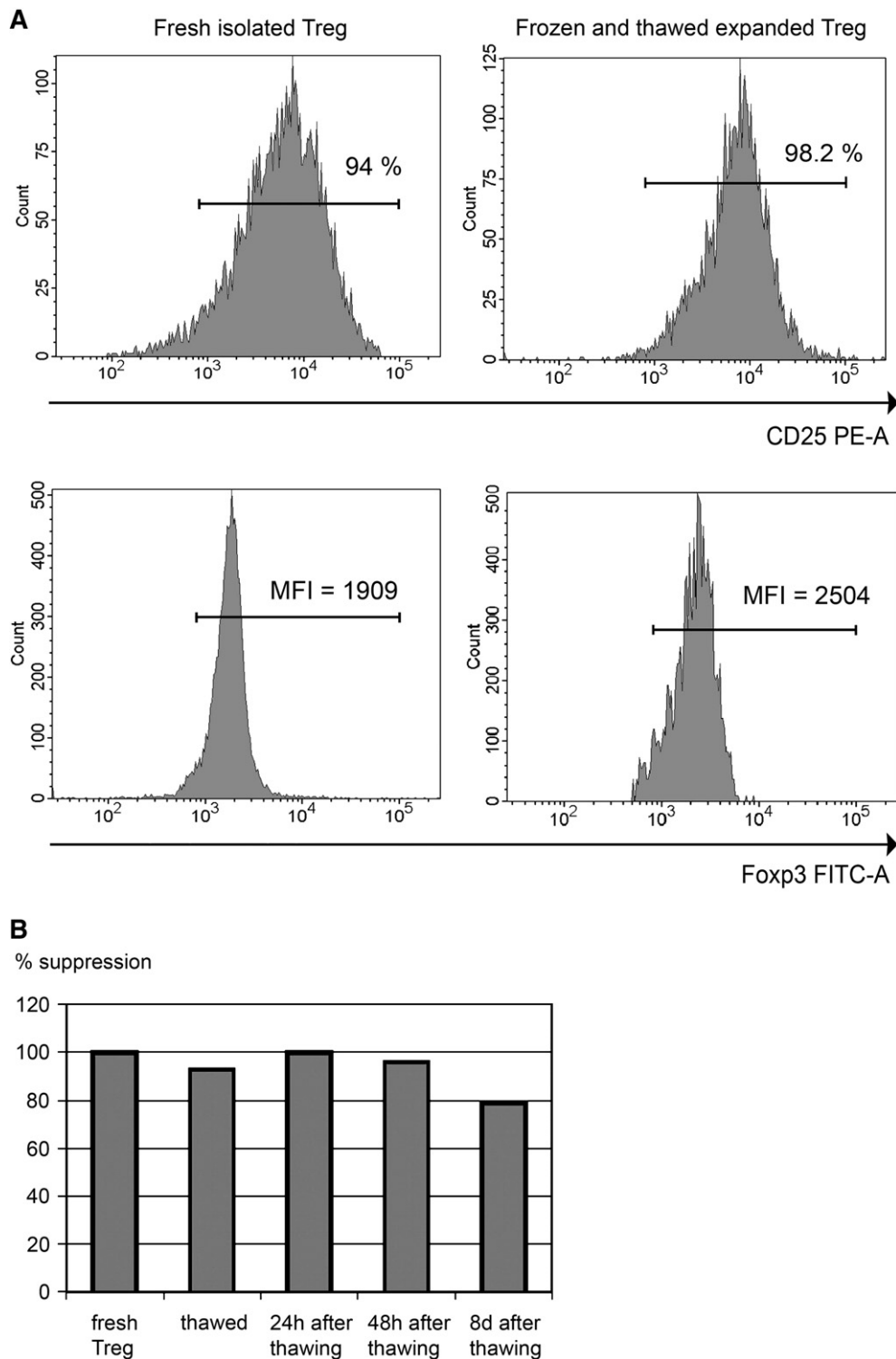


Fig. 4. Expanded Treg can be frozen and thawed without loss of function. Expanded Treg were frozen in liquid nitrogen on day 15 of culture. Compared to freshly isolated Treg (A, left panel) the thawed/expanded Treg (A, right panel) showed a comparable expression of CD25 (% of cells) and Foxp3 (mean fluorescence intensity). Functional assays using freshly isolated CD4⁺CD25⁻ effector cells stimulated with anti-CD3/CD28 and freshly isolated Treg or frozen/expanded Treg immediately after thawing, or 24 h, 48 h, 8 days after further expansion, demonstrated comparable suppressive capacity of the Treg after freeze/thawing (B). Shown are the results of one representative experiment out of three (A) or two (B) respectively.

Thus, these data demonstrate that the expanded Treg exert increased suppression in vitro by cell to cell contact and independently from IL-10 production.

3.4. Expanded Treg can be frozen and thawed without loss of function

On the basis of the high numbers of functional Treg obtained by expansion, we investigated the possibility of freezing and thawing of the expanded Treg, which would facilitate numerous experiments.

Therefore we froze aliquots of Treg after functional and phenotypical characterization. After thawing, cells were further expanded like described for freshly isolated Treg and subjected to FACS and suppression assays immediately after thawing and 1–8 days after re-culture. The FACS analysis showed that neither the expression of CD25 nor of Foxp3 was affected by freezing (Fig. 4A). Moreover, after subjecting the Treg immediately after thawing or up to 8 days in re-culture to functional in vitro assays with stimulated CD4⁺ responder T cells, we could still detect substantial suppression of the proliferation. Thus these data show, that freezing and thawing did not affect the regulatory function of the Treg (Fig. 4B).

3.5. Expanded and freeze/thawed Treg maintain their in vivo functions

So far our functional characterization relied on in vitro assays, however for future therapeutically applications it is essential to test the effects of expansion and/or freezing on the suppressive properties of Treg in vivo. We have previously shown (Ring et al., 2006) that injection of freshly isolated Treg leads to a significant suppression of the immune response in an experimental contact hypersensitivity (CHS) model. Therefore we applied this murine CHS model to expanded Treg. Here we show that i.v. injection of the same amount of freshly isolated or expanded Treg respectively (5×10^6 /mouse) resulted both in a significant suppression of the ear swelling reaction during CHS, which was even more pronounced using the expanded Treg (Fig. 5A). These results corroborate the data obtained in the in vitro assays showing that expanded Treg exert an increased suppressive capacity.

Applying the freeze–thawed Treg in vivo in the CHS model, we could further confirm that these cells retain their suppressive capacity after thawing as it has already been demonstrated in vitro (Fig. 5B). However, as compared to “freshly” expanded Treg, these cells showed a slightly reduced activity in vivo, though it was still superior compared to freshly isolated Treg. In summary these results

demonstrate, that Treg can be expanded and preserved by freezing without affecting their in vivo function.

Since these experiments were carried out with mixtures of LN and spleen derived Treg, we next analyzed whether the origin of the Treg has an effect on their suppressive activity in vivo. Therefore, we expanded Treg derived from LN and spleen respectively and injected these cells, or mixtures thereof, into mice before challenging with the respective hapten. As shown in Fig. 5C, we observed comparable suppression of LN and spleen derived Treg, however, the observed suppression was less effective when compared to mixtures of both cell types, thus indicating that LN and spleen derived Treg jointly support their suppressive function in vivo.

3.6. Freshly isolated and expanded T cells migrate to lymphoid organs irrespective of their origin

We have shown that in vitro expanded Treg exert an increased suppressive function, as compared to freshly isolated Treg. Furthermore, the origin of the cells is of importance, since mixtures of LN and spleen derived Treg exhibited a superior suppressive function. This is independent from IL-10 production or surface molecule expression. Yet another possible explanation for the different effects is that different migration pattern of the Treg populations in vivo affects their suppressive activity.

To compare the sites of action in vivo, we injected expanded and freshly isolated fluorescently-labeled Treg intravenously into the tail of sensitized mice followed by hapten challenging. At different time points, spleen and LN were excised and tested for presence of fluorescent cells. In the first 4 h after injection, we could detect an increasing amount of Treg in the spleen as well as in the LN (Fig. 6A). In addition, Treg accumulated faster in the spleens as compared to the LN and in the first 4 h, substantially more freshly prepared Treg entered the respective organs as compared to expanded Treg. However, in the further course of the experiment the Treg numbers in the lymphoid organs stagnated and only minor changes were recorded for the next 48 h and the differences were almost balanced 24 h after injection (Fig. 6B).

Since Treg have been shown to suppress T cell proliferation in vitro by cell to cell contact we further determined whether injected Treg migrate to the site of inflammation. To this end we also examined the ears by FACS analysis. These data (Fig. 6B) confirm our previous results, showing accumulation of Treg in lymphoid organs, but we could not detect any Treg in the inflamed tissue, the ear. Therefore we conclude that Treg suppress the elicitation phase of a CHS reaction independently from cell to cell contact in the inflamed skin.

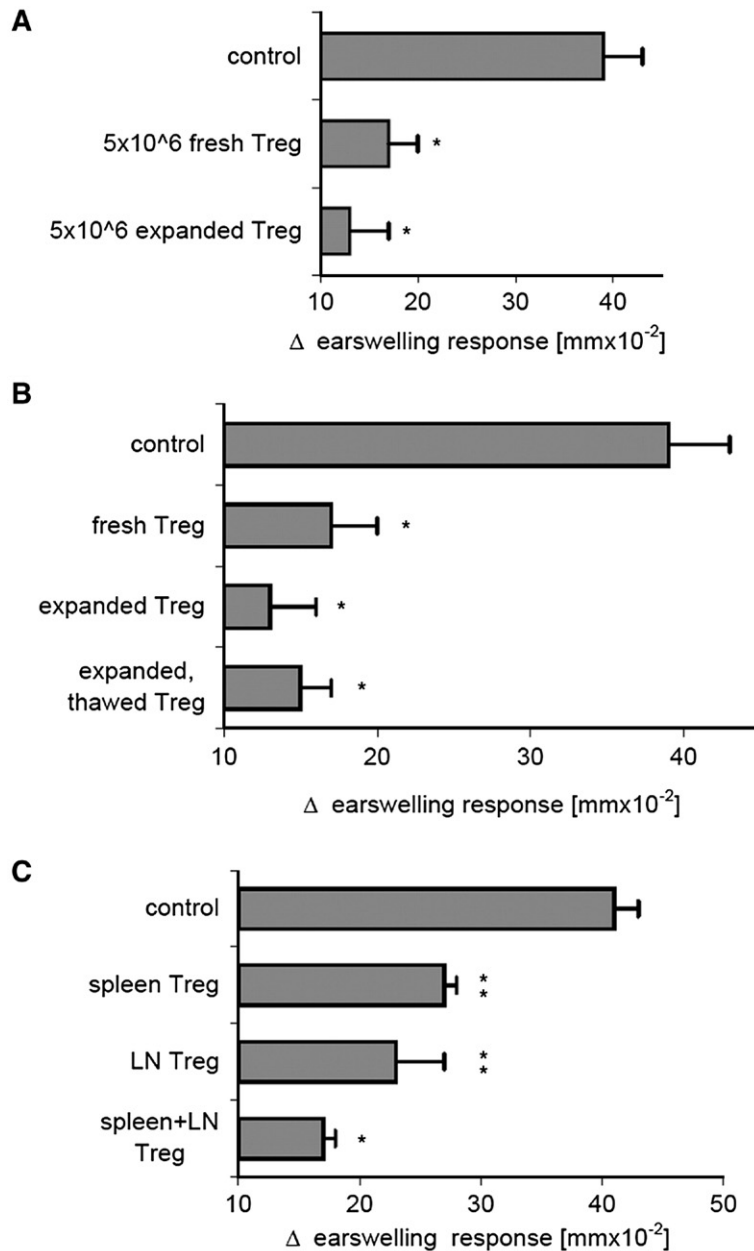


Fig. 5. Expanded and freeze/thawed Treg maintain their in vivo functions. BALB/c mice were sensitized with TNCB. Five days 5×10^6 freshly isolated Treg or expanded Treg were injected followed by application of TNCB to one ear. Control mice were not treated with cells at all. Ear swelling response was measured 24 h later. Herein, expanded Tregs showed an increased suppressive capacity in vivo compared to freshly isolated Treg (A). Freezing and subsequently thawing of the expanded Treg did not alter their in vivo function (B). Lymph node (LN) and spleen derived expanded Treg showed a better suppressive capacity in vivo than expanded Treg derived from either spleen or LN (C). Data shown represent the mean of the absolute increase of the ear thickness $\pm$ SD of five independent experiments (A+B) and of three independent experiments (C), each with five mice per group (* $p < 0.01$, ** $p < 0.05$).

Finally, in an attempt to investigate whether the origin of the Treg has an impact on homing, we generated Treg from LN and spleen and injected these dye labeled cells into animals. 24 h later the accumulation of the cells in LN and spleen was analyzed. Here

we show (Fig. 6D) that LN and spleen Treg home to the different organs in equal amounts. Moreover, the injection of a mixture of LN and spleen derived Treg had no effect on the distribution, thus the increased suppressive capacity of mixed Treg cultures on the CHS

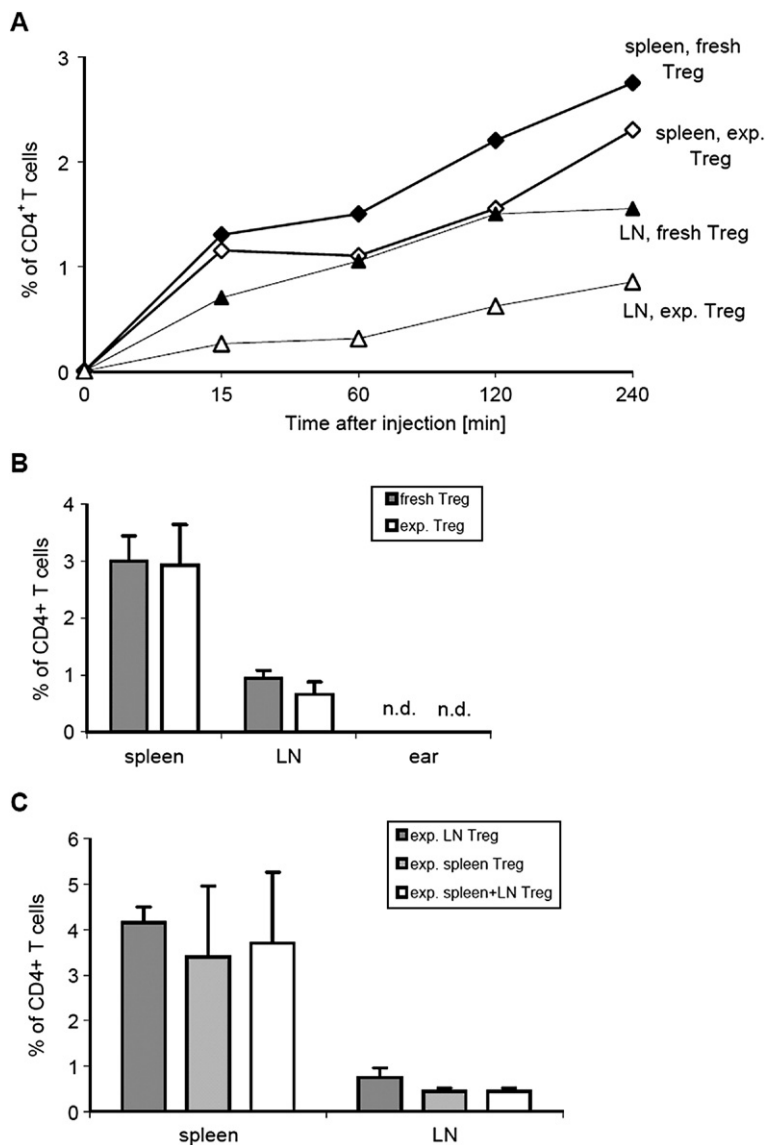


Fig. 6. Treg migrate to lymphoid organs irrespective of their origin. TNCB sensitized BALB/c mice were i.v. injected with PKH26-PE labeled freshly isolated or expanded Treg directly before challenge. After different time points (15 min–24 h) FACS analysis of draining lymph node (dLN) and spleen occurred. During the first 4 h after injection freshly isolated Treg migrated to a greater extent in the lymphoid organs than expanded Treg and both populations accumulated faster in the spleens as compared to the dLN (A). After 24 h the Treg numbers in the lymphoid organs stagnated and were almost balanced after this time in vivo (B). There were no injected cells detectable in the ear (B). The origin of the expanded Treg did not influence their migration pattern during the CHS reaction (D). Kinetic in A shows the result of one out of two experiments, each with two mice per group. B and C show the mean percent of injected PKH-PE labeled Treg within the endogenous CD4⁺ T cell population $\pm$ SD of five independent experiments each with three mice per group.

reaction is not attributed to different homing pattern to secondary lymphoid organs.

4. Discussion

The importance of CD4⁺CD25⁺ Foxp3⁺ regulatory T cells for the potential therapeutic use in autoim-

mune diseases, in graft-versus-host disease (GVHD) as well as after transplantation is becoming more and more apparent. The relatively small number of endogenous Treg that can be isolated from peripheral blood, restricts the in vivo application, leading to the development of several protocols to expand the Treg ex vivo (Takahashi et al., 1998; Tang et al., 2004;

Earle et al., 2005; Hoffmann et al., 2004; Godfrey et al., 2004).

Since one key-feature of Treg is their anergic state, the challenge in developing expansion protocols is to break this anergy without compromising the suppressive function. Therefore, most of the described protocols involve beads or antigen-presenting cells (APC) in co-cultures with the Treg, i.e., always “third party” cells are required, which demand complex cell culture techniques and complicate the further purification of the expanded Treg.

In the present study we established a convenient and efficient method to expand polyclonal murine Treg up to 180-fold within 3 weeks, maintaining their phenotype and even increasing their suppressive activity in vivo and in vitro. Other protocols using anti-CD3/CD28-coated microbeads together with IL-2 (Tang et al., 2004; Trenado et al., 2006) describe a more effective expansion, but the necessity to eliminate the beads from the cultures before using the Treg, implies an unnecessary stress for the cells.

APCs and DCs have also been identified as potent stimulators of Treg expansion. However, these methods are not as effective in breaking the anergy of Treg (Yamazaki et al., 2003; Fehervari and Sakaguchi, 2004; Brinster and Shevach, 2005) as our method and bear also the complication of re-isolating the Treg from the co-cultures. Thus, the anti-CD3/CD28 and IL-2 driven expansion of Treg as described herein yields pure Treg cultures, which can directly be used without any further manipulation.

On a functional level, the Treg expanded with DCs (Yamazaki et al., 2003) and the Treg expanded by anti-CD3/anti-CD28 differ in their capacity to produce IL-10. I.e., anti-CD3/anti-CD28 stimulation usually generates high levels of IL-10 in murine as well as in human cells (Tang et al., 2004; Levings et al., 2001) whereas DC expanded Treg lack this attribute. Thus, these data indicate that different subpopulations of CD4⁺CD25⁺ regulatory T cells may exist, which are distinct in their capacity to respond to different stimuli (i.e., DC versus CD3/CD28) and may convey their suppressive function cytokine-dependent or -independent, respectively.

Indeed, the efficiency of expanded and re-infused Treg has been proven during different autoimmune diseases in several murine models like autoimmune diabetes in NOD mice (Tarbell et al., 2004, 2007; Masteller et al., 2005), GVHD (Taylor et al., 2002; Trenado et al., 2006) and systemic lupus erythematosus (Scalapino et al., 2006). Nevertheless, the application of polyclonal expanded Treg is restricted to a limited number of severe diseases, since a generalized immunosuppression can not be excluded after injection of

these cells. Therefore, the use of DC expanded, antigen-specific regulatory T cells, which would target disease specific antigens, is not replaceable by signaling through the TCR and CD28 [reviewed in Masteller et al., 2006].

In addition to the mere expansion of Treg, we presented here for the first time that expanded Treg can be frozen and stored for weeks without losing their suppressive capacity. This offers the opportunity to generate Treg in large quantities and to use them as an “off-the-shelf reagent” for numerous applications.

With regard to studies with human Treg, Hoffmann et al. demonstrated an up to 40,000-fold expansion of highly purified human CD4⁺CD25^{high} T cells within 3 to 4 weeks when stimulated with cross-linked anti-CD3/anti-CD28 antibodies and high-dose IL-2. The possibility to preserve autologous Treg from patients by freezing may become a valuable tool for cellular therapies in the treatment of autoimmune diseases or GVHD.

Using our previously established model of contact hypersensitivity (Ring et al., 2006), we show that expanded Treg exert a higher suppressive capacity than freshly isolated Treg, which corroborates our in vitro results and is in line with several other reports. Thus, it is well documented that expanded Treg in general, regardless of whether they derive from mice or humans, display enhanced suppressive activity (Yamazaki et al., 2003; Hoffmann et al., 2004; Tang et al., 2004). However, the reason is not entirely clear, but in our study we show that the amount of Foxp3 on a per-cell basis expressed by the Treg increased significantly during the culture as compared to freshly isolated Treg. Beyond that, we could not detect any changes in cytokine production and surface marker expression, respectively. Therefore, our result strengthens the widely accepted notion that Foxp3 acts as the master control gene for the development and function of Treg (Hori et al., 2003; Khattri et al., 2003).

According to our data, preferential homing of expanded Treg to certain lymphoid organs, i.e., the organ of origin or the inflamed tissue, does not exist. Inherently naturally occurring Treg are able to re-circulate through lymphoid tissues as well as to enter inflamed sites, dependent on subset specific homing markers (reviewed in Huehn and Hamann, 2005).

However under different pathological conditions, the migration pattern of the examined Treg varies, indicating that the homing of Treg is critically dependent on the subset as well as on the experimental model and is consequently responsible for their respective function. Our data show, that in the CHS model, Treg, irrespective from their organ of origin, home to LN and spleen in similar shares. Since we detected the same migration pattern for the freshly isolated Treg, we assume that there were no changes in the

expression of specific homing markers during expansion procedure.

On the whole, our data indicate that Treg can be expanded and stored frozen without loss of function. In vitro as well as in vivo, these cells maintain their suppressive function and thus ex vivo Treg expansion may provide us with a novel tool to develop cell-based treatment regimens of allergic and autoimmune diseases.

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