 DOI 10.1002/eji.201141492 Eur. J. Immunol. 2011. 41: 3170–3175

European Journal of Immunology

SHORT COMMUNICATION

Peripherally induced human regulatory T cells uncouple Kv1.3 activation from TCR-associated signaling

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Peripherally induced Tregs (iTregs) are being recognized as a functional and physiologically relevant T-cell subset. Understanding the molecular basis of their development is a necessary step before the therapeutic potential of iTreg manipulation can be exploited. In this study, we report that the differentiation of primary human T cells to suppressor iTregs involves the relocation of key proximal TCR signaling elements to the highly active IL-2-Receptor (IL-2-R) pathway. In addition to the recruitment of lymphocyte-specific protein tyrosine kinase (Lck) to the IL-2-R complex, we identified the dissociation of the voltage-gated K^+ channel Kv1.3 from the TCR pathway and its functional coupling to the IL-2-R. The regulatory switch of Kv1.3 activity in iTregs may constitute an important contributing factor in the signaling rewiring associated with the development of peripheral human iTregs and sheds new light upon the reciprocal crosstalk between the TCR and the IL-2-R pathways.

Key words: Human Treg · IL-2 receptor pathway · Kv1.3 ion channel · Peripheral T-cell differentiation · T-cell signaling

Supporting Information available online

Introduction

Recent evidence supports the major role of peripherally induced regulatory T cells (iTregs) in controlling the immune response during inflammatory processes and against infectious agents [1–3]. The high degree of plasticity in the iTreg developmental program represents an additional challenge to the inherent difficulties associated with the study of human Tregs, such as insufficient number of cells, heterogeneous cell population and staggering differences between human and mouse models. These intrinsic limitations have prevented a comprehensive understanding of the differential signaling events that govern the development and function of Tregs. In the study presented here, we have taken advantage of a cell culture system that simultaneously generates functional populations of naïve, memory, effector T cells and iTregs from an original pool of primary human $CD4^+$ T cells. With this unique platform, we wanted to gain a better understanding of the signaling events that contribute to the altered proximal TCR-mediated activation of iTregs. A comparative analysis among T-cell subsets identified the uncoupling of voltage-gated Shaker family K^+-channel Kv1.3 activity from TCR engagement in iTregs. Kv1.3 has been implicated in the activation, migration, adhesion and volume regulation of human T cells [4]. Our findings demonstrate that, in

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iTregs, Kv1.3 and the reportedly functionally linked lymphocyte-specific protein tyrosine kinase (Lck) relocate to and participate in the signaling pathway triggered by the highly active IL-2-Receptor (IL-2-R) complex. We propose that the physical and functional redistribution of protein clusters, in addition to being instrumental to the altered TCR pathway in iTregs, may represent a common contributing mechanism to the signaling plasticity that governs T-cell lineage differentiation.

Results and discussion

Altered TCR signaling in iTregs

The physiological relevance of Tregs in the control of the immune response emphasizes the importance of understanding the molecular mechanisms underlying their development and function. In addition to naturally occurring, thymus-derived $CD4^+$ $CD25^{high}FoxP3^+$cells (nTregs), a microenvironment enriched with IL-2 and TGF-β can induce peripheral $CD4^+$ cells to differentiate into suppressor T cells in vivo and ex vivo [1, 2]. We have optimized culture conditions for $CD4^+$ $CD25^-$ primary T cells to allow the induction and discrimination of iTregs, naïve, memory and effector T cells. Cells that differentiate to a Treg-like phenotype also exhibit potent suppression of conventional $CD4^+$ T-cell proliferation (Supporting Information Fig. 1A). As expected, iTregs generated in our conditions had already acquired specific Treg signaling trademarks. Notably, iTregs exhibited a low activation of AKT in response to TCR engagement. In contrast, ERK activation was remarkably effective (Fig. 1A), indicating that the blockage of the AKT pathway in iTregs did not occur as a result of a general failure of the TCR signaling machinery. Therefore, we propose that the differentiation to iTregs involves the rewiring of the signaling network associated with TCR-dependent events upstream of the AKT pathway.

Functional dissociation between Kv1.3 and TCR activation in iTregs

Previously, we demonstrated that, in contrast to conventional $CD4^+$ and $CD8^+$ T cells, the activation of the K^+ channel Kv1.3 was refractory to TCR stimulation in human nTregs [5]. To explore whether iTregs displayed the same functional trait along with the acquisition of other regulatory/suppressor trademarks, we analyzed and compared the response of Kv1.3 to TCR engagement among different T-cell subsets. Using an automated, high-throughput patch-clamp screening assay, we found a similar profile of weak Kv1.3 response to TCR stimulation in induced and natural Tregs (Fig. 2A). Based on the observation that the mRNA and protein expression of Kv1.3 remained steady compared to effector cells (Fig. 2B), these findings suggest that the functional dissociation between TCR and Kv1.3 activity is likely due to the remodeling of the TCR signaling network during iTreg differentiation.

Regulation of Lck activity by IL-2-R pathway in iTregs

The association between the Src-family tyrosine kinase Lck and Kv1.3 is necessary for the initiation of the TCR-mediated signaling triggered during the formation of the immune synapse [6–8] and has been reported in other, non-TCR-dependent, T-cell responses as well [9–11]. Therefore, we explored whether a differential Lck activation in iTregs may concur with the loss of control of Kv1.3 activity by TCR. In our experimental setting of comparative Western blot analysis depicted in Fig. 1A, iTregs exhibited the highest levels of active Lck in mock-treated samples under normal resting conditions (2 h on ice), as indicated by the intense phosphorylation of Lck at Y394. In spite (or because) of this high basal activity, iTregs displayed a reduced capacity to increase Lck activation in response to TCR ligation. A phenotypic attribute of iTregs is the high expression of CD25, the IL-2-Rα subunit that associates with the IL-2-Rβ (CD122) and the common γ (CD132) chains to form the high-affinity IL-2-R complex. In conjunction with the requirement for a constant supply of IL-2, the enhanced expressionn of the high-affinity IL-2-R complex underscores the critical role that the IL-2/IL-2-R pathway plays in iTreg development [12, 13]. In this context, the fact that Lck exhibited a similarly enhanced activity in iTregs when the TCR signaling machinery was not triggered suggests that an alternative pathway might activate Lck. In order to assess the potential involvement of the highly active IL-2-R, we performed co-immunoprecipitation assays of Lck from effector T cells and iTreg lysates. The results in Fig. 3A confirmed that the physical association between Lck and the IL-2-Rβ subunit occurred only in iTregs. In addition, we generated evidence that Lck is activated upon IL-2-R engagement in iTregs. Sorted iTregs rested for additional period of time (6–7 h on ice) revealed the activation of TCR-dependent downstream events (as depicted in Fig. 3B by the phosphorylation of LAT), although the actual level of activated Lck was essentially identical for TCR crosslinked and mock-activated cells. Conversely, a marked increase of Y394-phosphorylated Lck was detected in iTregs treated with IL-2 that paralleled the IL-2-R-induced phosphorylation of STAT5. In agreement with others [14, 15], these findings support the direct involvement of Lck in the IL-2-R signal transduction pathway of iTregs. Since we observed no differences in Lck expression between conventional T cells and iTregs (Figs. 1B, 1C and 3A), these results are consistent with the redistribution of Lck to the IL-2-R cluster occurring during the development of iTregs.

Regulation of Kv1.3 function by IL-2-R pathway in iTregs

We next wanted to address the question of whether the relocalization and clustering of Lck to the IL-2-R complex in iTregs encompassed the regulatory switch of Kv1.3 activity. The functional incidence of Kv1.3 spatial redistribution has been already documented in T cells [6–11]. In order to examine whether the TCR-dissociated Kv1.3 becomes functionally linked to the highly active IL-2-R complex, we performed

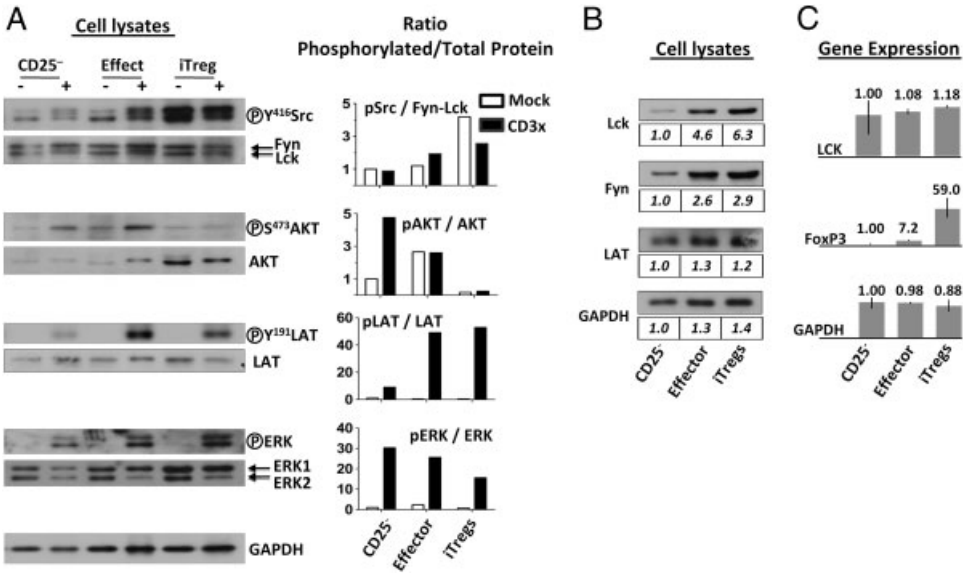

Figure 1. TCR-dependent signaling in iTregs. (A) Sorted $CD4^+CD25^-$ T cells, effector T cells and iTregs were rested for 2 h on ice and activated (+) or not (–) by anti-CD3 crosslink for 3 min. Lysates of 5×10^5 cells were analyzed by Western blot to detect activated (phosphorylated) forms and total protein expression of Lck/Fyn (anti-pY416-Src recognizes both pY394-Lck and pY417-Fyn), AKT, LAT and ERK. GAPDH expression demonstrated equivalent loading. Protein band densities were quantified by densitometry. Ratio of phosphorylated:total protein was normalized to that of mock-treated $CD25^-$ cells and depicted as mock-treated (empty bars) or CD3 crosslinked (black bars) cells. (B) Lysates from 5×10^5 cells were analyzed by Western blot to detect the pattern of expression of proteins involved in early events of TCR signaling. Protein band densities were quantified and normalized to that of $CD25^-$ cells as indicated below each blot. (C) Transcription of the LCK gene was quantified from $CD25^-$ T cells, effector T cells, and iTregs. FOXP3 (as a positive control) and GAPDH (housekeeping gene) were assessed as control. Briefly, total RNA was extracted from 5 to 7×10^6 sorted cells. cDNA was obtained by reverse transcription and used as a template in a microarray analysis with the Affymetrix GeneChip System (Human Genome U133 Plus 2.0 Array microchip). The results represent the comparative mean value of triplicates from three different donors relative to $CD25^-$ samples; bars represent the experimental range of the triplicates. The results were validated with real-time PCR (not shown).

high-throughput patch clamp analysis with sorted iTregs incubated with IL-2, IL-6 or IL-10. Figure 3C shows that, in contrast to the weak response observed upon TCR ligation, iTregs in culture with IL-2 sustain significantly larger Kv1.3 peak currents compared with cells cultured with IL-6 or IL-10. These results provide, to our knowledge, the first evidence of the functional integration of Kv1.3 into the IL-2-R network in human T cells. Moreover, the linkage of Kv1.3 to the highly Lck-activating IL-2-R coincides with the functional uncoupling from TCR control. The enzymatic activity of Lck is required to initiate the TCR signaling cascade that regulates T-cell development, differentiation and activation [16]. However, despite extensive investigation, the precise mechanistic principles that orchestrate the earliest signaling events induced by Lck are still elusive. Recent reports propose that a pool of pre-activated-Lck gains access to the ITAM sequences within the CD3/TCRζ complex exposed by the conformational changes upon TCR engagement [17, 18]. According to this model, rather than the activation of Lck, the critical priming step in the initiation of the TCR pathway would be the substrate accessibility (i.e. CD3 and TCRζ cytoplasmic tails) to the pool of available Lck. Haughn et al. [19] demonstrated that a very active, high-affinity IL-2-R in T cells might cause the functional uncoupling of the TCR signaling machinery through diversion of the subcellular localization of Lck to the IL-2-R multiprotein. Accordingly, the strong activation of Lck by the IL-2-R pathway in iTregs may compromise the amount of Lck available in CD3/TCR complexes and therefore alter the TCR signaling cascade. In agreement with other reports [7–9], our findings also suggest that Kv1.3 remains functionally linked with the pool of Lck susceptible to lateral mobility. Interestingly, the differentiation to iTregs occurs preferentially in cells that undergo strong proliferative expansion (not shown), which involves continuous TCR-mediated regulation of Kv1.3 function and requires a strong activation of the AKT pathway. During the transition to the differentiation stage and the acquisition of the suppressor phenotype, the formation of new protein complexes occurs concomitantly with the remodeling of functional signaling paths that eventually dissociate AKT and Kv1.3 activities from the TCR network. We provide evidence that uncoupling early TCR signaling elements occurs, in addition to other potential mechanisms [20], through the competitive crosstalk between TCR and IL-2-R pathways in cells with high IL-2-dependent activity. These findings support a dynamic model by which the redistribution of common, key signaling components (Lck-Kv1.3) may represent a rapid and efficient mechanism of adapting the cell signaling machinery to a new environmental context. The functional switch from antigen-dependent signaling in effector cells to cytokine-dependent responsiveness in iTregs is consistent with the physiological prevalence of the suppressor activity of iTregs upon antigen clearance.

Concluding remarks

Collectively, these results: (i) provide a novel mechanistic link between the remodeling of a signaling network and the acquisition of suppressor iTreg phenotype with the selective relocation of TCR-associated proximal components; (ii) support the importance of the finely tuned TCR/IL-2-R crosstalk in the control of T-cell fate decisions and (iii) underscore the differential role that K^+ channels may play in specific T-cell subpopulations and/or their functional responses.

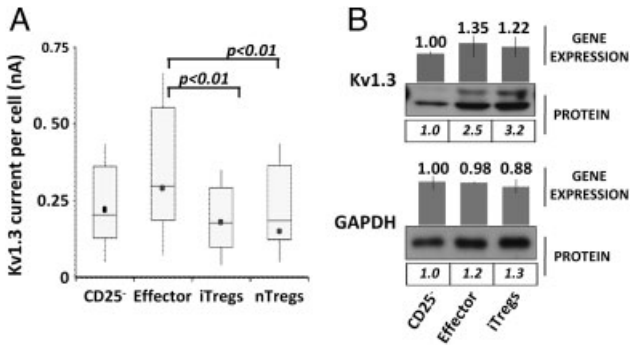

Figure 2. Kv1.3 in iTregs. (A) $CD4^+CD25^-$ T cells and nTregs isolated from the same donors were cultured in iTreg medium (Supporting information). After 6 days, $CD4^+CD25^-$ T cells, effector T cells, and iTregs were sorted and maintained for 60 h in serum-free AIMV medium with or without 0.15 μg/mL of soluble anti-CD3 antibody. Kv1.3 current in sorted cells and nTregs was measured with high-throughput patch-clamping screening. Experimental values represent the combined distribution of Kv1.3 measurements on individual cells isolated from three independent experiments. $N = 255$ $CD4^+CD25^-$ cells; $N = 186$ effector cells; $N = 88$ nTregs; $N = 129$ iTregs. The distribution of Kv1.3 values is depicted in box plots with whiskers representing the 10th and 90th percentiles of experimental values; boxes are limited by the 25th and the 75th percentiles; solid lines are the medians, and dark squares represent the mean values. A two-sample Kolmogorov–Smirnov test was used to compare paired distributions of Kv1.3 currents. (B) Sorted $CD4^+CD25^-$ T cells, effector T cells, and iTregs were analyzed by Western blot to compare the expression of Kv1.3. The double-banded pattern depicted in effector and iTreg Kv1.3 blots may likely correspond to different glycosylation forms of Kv1.3 [23]. Quantification of protein expression was normalized to the band density of $CD25^-$ cells. GAPDH levels demonstrate equal protein loading. Gene expression was determined as detailed in Fig. 1C.

Materials and methods

Cell isolation, culture conditions and suppression assay are detailed in Supporting Information.

Buffer compositions

In total, 100 mM K^+-D-gluconic acid, 50 mM KCl, 3 mM $MgCl_2$ and 5 mM EGTA pH 7.3 was used as intracellular recording solution in the patch-clamping. Radio-Immuno-Precipitation Assay (RIPA) buffer: 50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 0.1% SDS, 0.5% Na-Deoxycholate and 1% Triton X-100. Immunoprecipitation lysis buffer: 50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% Nonidet P-40 and 0.5% *n*-dodecyl-β-D-maltoside. Lysis buffers were supplemented with 1 mM PMSF and Halt Protease inhibitor cocktail (Pierce).

T-cell activation and lysates

Cells were stimulated with anti-CD3 (0.5 μg/10^6 cells) cross-linked with anti-mouse IgG (1.2 μg/10^6 cells) for 3 min at 37°C or incubated with IL-2 (5 ng/mL) for 6–7 h. Later, cells were lysed for Western blotting or immunoprecipitation.

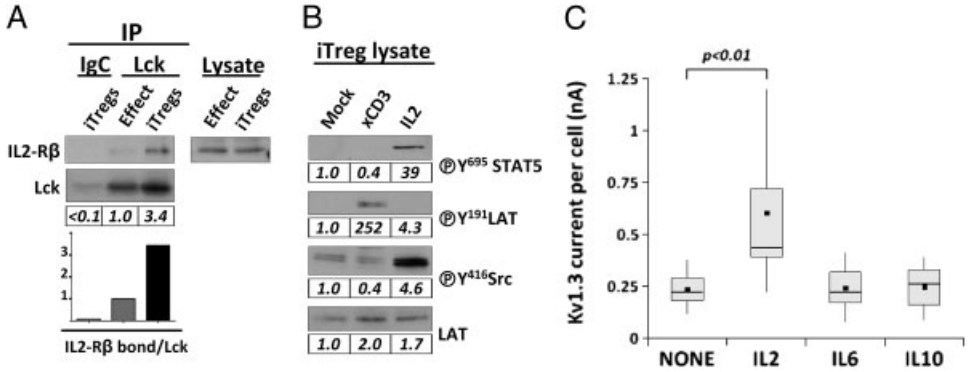

Figure 3. Lck and Kv1.3 respond to IL-2 in iTregs. (A) Lysates from 10^7 effector T cells and iTregs were incubated with Lck antibody-loaded agarose beads or Ig-isotype control (IgC). Cell extracts and immunoprecipitates were analyzed by SDS-PAGE and Western blots using CD122 (IL-2-Rβ) and Lck antibodies. Quantification of co-immunoprecipitates was determined by the relative ratio of IL-2-Rβ bound to Lck and normalized at 1.0 in effector cells. (B) Sorted iTregs were rested for 6–7 h on ice and mock-activated, anti-CD3 crosslinked or IL-2 treated. Lysates of 5×10^5 cells were subjected to immunoblotting using the indicated antibodies. LAT expression verified equal loading. Quantification of protein expression was normalized on the basis of mock-treated iTregs for each individual blot. (C) Sorted iTregs were cultured for 60 h in AIMV with or without 5 ng/mL of IL-2, IL-6 or IL-10. Depiction and measurement of Kv1.3 currents were performed as described in Fig. 2. Profiles of Kv1.3 currents represent the combined values of two independent experiments. A two-sample Kolmogorov–Smirnov test was used to compare paired distributions of Kv1.3 currents.

Western blotting, immunoprecipitation and autoradiogram

Western blots were analyzed with the following antibodies: p-Tyr, Lck, Zap70, LAT, AKT, ERK 1/2, CD122 and GAPDH from Santa Cruz; from Cell Signaling p-Src; Zap70 and p-Zap70; STAT5 and p-STAT5; p-LAT; p-AKT; p-ERK 1/2. Kv1.3 antibody (Alomone labs). Lck antibody-conjugated agarose (Santa Cruz) was used to immunoprecipitate cell lysates. Details of Lck immunoprecipitation and western immunoblottings were described elsewhere [21]. Densitometric analysis with the ImageQuant v.5.1 software (Molecular Dynamics) was used to quantify the intensity of labeling. Pixel densities for each band were normalized within the same experiment and autoradiography exposure.

Electrophysiology

We used the automated IonWorks HT high-throughput patch-clamping system (Essen Instruments, Ann Arbor, MI, USA) as a screening platform to determine Kv1.3 current profiles in different T-cell subsets and culture conditions. It is worth noting that the IonWorks HT device cannot screen for cell capacitances, therefore we cannot estimate frequencies of the current density [22]. Details on data protocols, conditions, processing algorithms and validation of this methodology are described elsewhere [5].

Statistical analysis

The Kruskal–Wallis ANOVA test was used to determine differences in peak currents among multiple groups. Statistical comparisons of current distributions between two samples were performed using the Kolmogorov–Smirnov test. Differences were considered significant when $p<0.01$.

Acknowledgements: The authors thank Jennifer Strange and Greg Bauman for their assistance with Flow Cytometry analysis and sorting. This work was supported by NIH Grant Numbers R03AR052904-02 from the NIAMS and 2P20 RR020171 from the NCRR to F. M.; D. J. E. acknowledges a fellowship from the Cellular Biotechnology Training Program (CBTP) from NIH and a Rackham Pre-Doctoral Fellowship from the University of Michigan.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **nTreg:** natural Treg · **iTreg:** induced Treg · **IL-2-R:** IL-2-Receptor · **Lck:** lymphocyte-specific protein tyrosine kinase · **AKT:** protein kinase B · **LAT:** linker of activated T cells

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Received: 8/2/2011
Revised: 8/6/2011
Accepted: 8/8/2011
Accepted article online: 11/8/2011

