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Selective targeting of transforming growth factor-beta1 into TCR/CD28 signalling plasma membrane domains silences T cell activation

Thomas Harder^{*}, Karina Guttek, Lars Philipsen, Luca Simeoni, Burkhard Schraven and Dirk Reinhold

Abstract

TGFβ1 (Transforming Growth Factor-beta1) is a versatile regulator of T cell immune responses. Depending on its context in the immunological environment, TGFβ1 guides T cells toward specific activation programs including T_H17 and regulatory T cell activities. Moreover, TGFβ signals function in immune homeostasis by directly attenuating T cell effector activities. We uncovered a novel context under which TGFβ1 stringently and reversibly silences activation responses of resting human T cells to TCR/CD28 stimulating surfaces:

Using ligand-presenting beads, TGFβ1 and TCR/CD28-activating signals were directed into defined plasma membrane domains of T cells. Selective targeting of TGFβ1 cytokine into TCR/CD28 signalling plasma membrane domains held back early response of TCR-proximal tyrosine phosphorylation and bead engulfment at activation sites. Consequently, downstream induction of proliferation and cytokine secretion were stringently attenuated. After extended incubation with TGFβ1-presenting beads, silenced T cells became receptive again to activation by renewed TCR/CD28-stimuli, indicating that the unresponsive state of T cells was reverted and did not reflect long-lasting anergy or decrease in T cell viability. These findings outline a new strategy of physically linking TGFβ1 and TCR-activating functions for the treatment of disease and pathological conditions which are caused by unwanted T cell activity.

Keywords: Silencing of T lymphocyte activation, TGFβ1, Plasma membrane compartmentalisation, Biologicals for treatment of transplant rejection, Allergies, Autoimmunity

Lay abstract

The human body is permanently exposed to pathogens like viruses which infect cells of the body and cause disease. In order to control pathogen attack, humans and animal species have evolved a sophisticated defence; the immune system. The immune system needs to recognise pathogens, fend them off, and memorise these pathogens in case they reappear. However, loss of control over these defence mechanisms is a cause of many serious diseases like diabetes, inflammatory bowel disease, multiple sclerosis, and allergies. In the present study we developed a set-up under which key cells of the immune system; so-called T cells, are held in a dormant state under which they do not activate their functions in immune defence. This new route of T cell-silencing promises a lead toward

treatment of numerous important diseases which are caused by unwanted action of T cells like diabetes, inflammatory bowel disease, multiple sclerosis, and allergies.

Background

Transforming growth factor-beta (TGFβ) has pleiotropic roles in steering differentiation and homeostasis of cells, tissues, and organs [1]. TGFβ1 is a versatile regulator of T cell immune responses and guides CD4⁺ T lymphocytes toward specific activities of the T_H17 and Treg phenotypes [2,3]. Additionally, TGFβ signals restrain T cell activities and thereby directly function in immune homeostasis [4,5]. Functions of TGFβ1 in safeguarding a healthy balance of the immune system were highlighted by severe multiorgan inflammatory disease of TGFβ1-deficient mice [6,7]. Dysfunction of TGFβ1-mediated T cell balance is associated with autoimmune disorders and thus molecular

* Correspondence: tharder@faciendum.org
Institute for Molecular and Clinical Immunology, Otto-von-Guericke University Magdeburg, Leipziger Straße 44, D 39120 Magdeburg, Germany

principles of TGF β -regulation of T cell activities are of important medical interest.

TGF β signals are transduced by TGF β receptors (TGF β R) I and II which catalyse serine/threonine phosphorylation of SMAD proteins. Phosphorylated SMADs cooperate with various transcription factors to activate cell-specific gene transcription programs. TGF β receptors, cytoplasmic and nuclear factors, and chromatin structure determine context-dependent cellular outcomes of these canonical TGF β signalling cascades. Additional non-canonical TGF β signalling pathways exist, many of which are initiated at the platform of the plasma membrane for example by targeting PI3K/AKT pathway and Rho-GTPase signalling cascades [1].

Under physiological, resting conditions TGF β is held in an inactive latent configuration in a complex with Latency Associated Peptide (LAP) which hinders TGF β cytokine binding to TGF β receptors. Latent TGF β gets activated when active cytokine is released from TGF β -LAP complex. Various mechanisms mediate activation of TGF β : These include specific binding of proteins like thrombospondin to LAP, LAP proteolytic cleavage, α integrin-binding and cytoskeletal force along membrane-bound TGF β -LAP complex [8-10].

T cell antigen receptor (TCR), upon interaction with a cognate peptide-MHC ligand, transduces key signals which activate resting T lymphocytes to proliferate and perform immune effector functions. These signals are propagated to the T cell interior via tyrosine kinase activation and induction of signalling complexes which mediate intracellular signalling reactions and respective T cell responses [11]. A costimulatory second signal is mediated by surface protein CD28 on engagement with CD80/86 antigens on antigen-presenting cells and is required for full activation of resting T lymphocytes to proliferate and perform effector functions like secretion of cytokines [12].

TGF β 1-LAP is presented on the surface of CD4+ CD25+ T cells and was proposed to exert regulatory functions upon contact with effector T cells [13,14]. On FoxP3+ regulatory T cells (Tregs) TGF β 1-LAP is bound to trans-plasma membrane protein GARP (glycoprotein A repetitions predominant protein) [15,16]. siRNA-mediated depletion of GARP in human Tregs reduced, but did not abolish, suppressive activity of FoxP3+ Tregs *in vitro*, indicating contribution of TGF β 1 surface presentation as well as additional functions to regulatory activities of Tregs [16]. A distinct population of FoxP3+ human Tregs expresses MHC class II and was shown to specifically mediate early suppression of T cell activation directly upon cell/cell contact [17].

TCR activation and TGF β 1-signals may likewise act together at the immunological synapse between CD4+ T cells and antigen-presenting dendritic cells (DC) in order

to induce Treg and T_H17 activities. These DCs need to express matching MHC class II to activate TCR and α integrins which present TGF β 1 to T cells [18-20].

Hence, simultaneous presentation of TGF β 1 and TCR-activating ligands on cell surfaces is a recurring motif in modulation of T cell activities. Here we used defined ligand-coated beads as artificial surfaces to present activating stimuli to resting human T cells *in vitro*: Simultaneous presentation of TGF β 1 and TCR/CD28-signals in common T cell plasma membrane signalling domains stringently and reversibly held back activation responses of T cells. These findings identified lateral plasma membrane compartmentalization of TGF β 1 signalling as cell biological context under which T cell activation responses are attenuated and suggest new strategies for treatment of disease and pathological conditions which are caused by unwanted T cell activity.

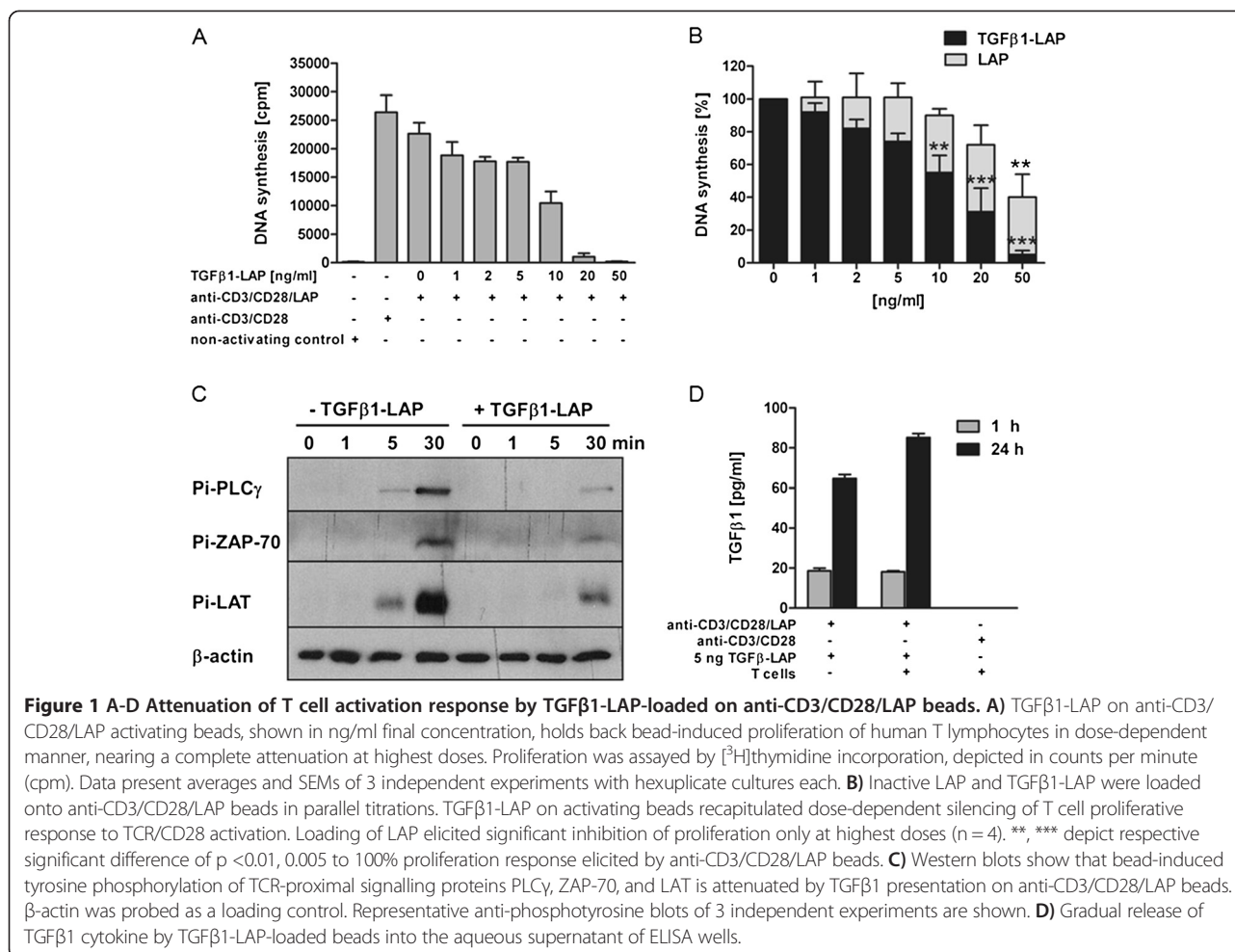
Results and discussion

Presentation of TGF β 1 on TCR/CD28-activating beads silences T cell activation responses

Isolated resting human T lymphocytes were activated via TCR/CD28 by conjugation with protein G-coated microbeads which were coated with anti-CD3 and anti-CD28 mAbs (monoclonal antibodies) and, additionally, with mAb against the LAP subunit of latent TGF β -LAP complex. Defined amounts of TGF β 1-LAP were loaded onto anti-CD3/CD28/LAP beads via anti-LAP antibodies. TGF β 1-LAP-loading silenced proliferative response of T cells to bead-bound anti-CD3 and anti-CD28 mAbs in strictly dose-dependent manner; first evident around 2 ng/ml final concentration and nearing completion at 50 ng/ml (Figure 1A).

We estimated contribution of steric impediment of antibody access to T cells by bead-bound TGF β 1-LAP to attenuation of T cell proliferation response. In parallel experiments TCR/CD28-activating beads were loaded with inactive LAP and with latent TGF β 1-LAP. Inactive LAP, when loaded on T cell-activating anti-CD3/CD28/LAP beads, partially inhibited proliferation at highest doses (Figure 1B) indicating that steric impediment of activating antibody access contributes to the inhibitory effect. This, however, can be discriminated from strictly dose-dependent and stringent antiproliferative activity of TGF β 1-LAP on activating beads.

We then assessed whether presentation of TGF β 1-LAP on anti-CD3/CD28/LAP beads also attenuated early response of tyrosine phosphorylation of TCR-proximal signalling proteins [21]. Western blots of whole cell lysates of TCR/CD28 activating bead-stimulated T cells were performed over a time course of 30 mins, 37°C incubation. These blots reiterated TCR triggering-induced tyrosine phosphorylation of tyrosine kinase ZAP-70, transmembrane Linker for Activation of T cells (LAT)



and phospholipase C γ (PLC γ), with respective Mw at 70 kD, 36 kD and 110 kD (Figure 1C). Importantly, when TGFβ1-LAP was presented by TCR/CD28-activating beads, tyrosine phosphorylation of ZAP-70, LAT, and PLC γ was reduced. These data suggest that silencing of T cell activation responses upon TGFβ1-LAP presentation on TCR/CD28-activating beads commences with attenuation of early TCR-proximal tyrosine phosphorylation.

Holding back T cell activation response by local presentation of latent TGFβ1-LAP implied mechanisms which release active TGFβ1-cytokine toward TCR/CD28 activation domains. ELISA analysis of bead supernatants indicated spontaneous release of TGFβ1-cytokine from beads at a slow rate (Figure 1D) which may be sufficient for the T cell-silencing mechanism described here. Presence of T cells elicited an additional ~30% release of TGFβ1 at 24 h 37°C incubation. Association/dissociation kinetics of TGFβ-LAP complexes and of TGFβ with TGFβRI and II were comprehensively characterized in plasmon resonance studies [22], unravelling rates of spontaneous slow dissociation and rapid rebinding of TGFβ1 to LAP, consistent with our results. In our ELISA assays

bead-bound non-complexed LAP molecules compete with TGFβ-capturing ELISA antibodies for binding free TGFβ1 cytokine. Moreover, T cellular activities partially contribute to latent TGFβ1-LAP activation. In conclusion, our ELISAs demonstrated gradual release of active TGFβ1 cytokine from beads but could not estimate quantities and rates in T cell/bead-conjugates.

TGFβ and TCR/CD28 signals cooperate in common plasma membrane domains in order to silence T cell proliferative response

We next queried the requirement of simultaneously presenting TCR/CD28 activating stimulus and TGFβ1 activity on the same bead for holding back T-cell proliferation (Figure 2AB). This was addressed by presenting anti-CD3/CD28 stimulus and TGFβ1-LAP on separate beads (Figure 2A). TGFβ1-LAP was presented by protein G beads which were either coated with anti-LAP antibodies alone or with anti-LAP and anti-CD28 antibodies at a 1:1 ratio in order to enhance contact of anti-LAP beads with T cells. Importantly, both ways of presenting TGFβ1-LAP to T cells had little influence on proliferative response

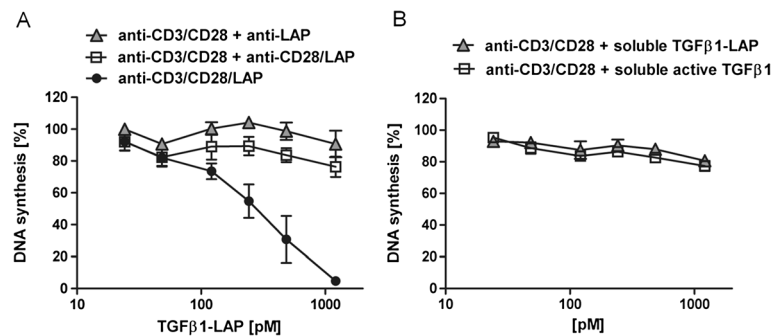


Figure 2 A, B Selective targeting of TGFβ1 into TCR/CD28 activation sites attenuates T cell proliferative response. A) TGFβ1-LAP, when presented to human T lymphocytes by anti-LAP- (grey triangles) or anti-LAP/CD28- (white squares) antibody-coated protein G beads, does not inhibit proliferative response elicited by separate anti-CD3/CD28 antibody-coated beads (n = 3). TGFβ1-LAP presented on anti-CD3/CD28/LAP beads (black circles) effectively held back proliferation (n = 4). **B)** Soluble TGFβ1, as latent TGFβ1-LAP (black circles) or active TGFβ1 cytokine (white squares), was present in equimolar amounts as TGFβ1-LAP for Figures 1A, 2A and did not effectively hold back T cell proliferation induced by activating anti-CD3/CD28 beads.

induced by anti-CD3/CD28 beads. This indicated that stringent silencing of T cell proliferative response only occurred when TCR/CD28 activating stimuli and TGFβ1-LAP were presented to T cells together on the same bead.

TGFβ1 was presented to T cells as soluble latent TGFβ1-LAP complex or as soluble active TGFβ1 cytokine and elicited little antiproliferative effect on T cells which were activated by anti-CD3/CD28 protein G beads (Figure 2B). It is particularly important to emphasise that, at this specific setting of T cell activation, soluble active TGFβ1 cytokine could not substitute for the lack of TGFβ1 presentation on T cell activating beads in blocking activation response; suggesting that TCR/CD28- and TGFβ-signals integrate and cooperate in common T cell plasma membrane domains and then provide signals which silence T cell activation response. Interestingly, MHC class II expression identifies a distinct population of human Tregs which mediates early suppression of effector T cell activation responses on direct cell/cell contact [17]. It is tempting to speculate that silencing beads mimic suppressive activities of this MHC class II + Treg population in the human system.

Diminished proliferative response of bead-silenced T cells to TCR/CD28-activating culture plates

We observed that TGFβ1 elicits potent inhibition of T cell proliferative response when presented in physical proximity of activating TCR/CD28 stimuli. We sought to characterize response of these TGFβ1-silenced T cells when activated by fresh, independent TCR/CD28 stimulus (Figure 3AB). First, T cells were silenced for 15 min at 37°C with TGFβ1-LAP on anti-CD3/CD28/LAP beads (Figure 3A). T cell/bead conjugates were then transferred to tissue culture plates which were either left untreated or were coated with anti-CD3 and anti-CD28 antibodies.

After 3 days on plates T cell proliferation rates were determined. ³H-thymidin incorporation was used to measure T cell proliferation rates. On transfer to uncoated non-activating plates proliferation was induced by activating beads without TGFβ1-LAP loading at 15635 ± 9310 cpm SEM and was taken as 100% proliferative response value. Variation and relatively low proliferation rates may be caused by variable loss of T cell/bead contact on transfer. Dose-dependent and stringent antiproliferative action of bead-bound TGFβ1-LAP, however, was always recapitulated. Transfer of 15 min-incubated T cells/beads to anti-CD3/CD28-coated tissue culture plates strongly enhanced T cell proliferation rates. Proliferative response to plate-bound anti-CD3/CD28-activating stimulus was significantly attenuated to ~31% by TGFβ1-LAP presentation on initial activating beads. These results indicate that T cells which were silenced by anti-CD3/CD28/LAP bead-presented TGFβ1 for 15 min exhibited a reduced response to fresh TCR/CD28 activating surface.

Next, initial bead/T cell activation reactions were incubated for 24 h before transfer to tissue culture plates (Figure 3B): On transfer to uncoated plates bead-induced proliferation without TGFβ1-LAP was measured at 47881 ± 12792 cpm SEM. Again proliferation of T cells was inhibited by TGFβ1-LAP loaded on TCR/CD28-activating beads (Figure 3B). Here however, TGFβ1-mediated suppression of T cell proliferation was less pronounced than for bead/T cell conjugates after 15 min preincubation in Figure 3A. This suggested that after 24 h of preincubation silencing activity of TGFβ1-LAP-loaded TCR/CD28-activating beads was reduced, and that silenced T cells reverted to be receptive to renewed proliferative stimulus by fresh contact with bead-bound anti-CD3/CD28 antibodies on transfer. Indeed, 24 h-preincubated T cells responded to anti-CD3 and anti-CD28 on plates with little

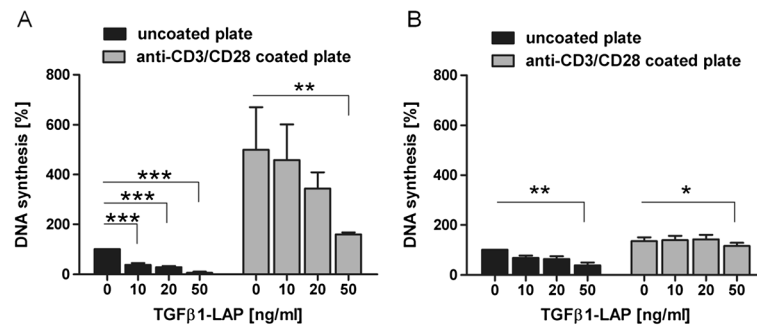


Figure 3 Attenuated proliferative response of bead-silenced T cells to TCR/CD28-activating culture dishes. T cells were incubated in round-bottom tubes with anti-CD3/CD28/LAP beads which were loaded with indicated amounts of TGFβ1-LAP. After **A)** 15 min and **B)** 24 h bead/T cell activation reactions were transferred onto tissue culture plates which were either left untreated (black bars) or were coated with anti-CD3 and anti-CD28 antibodies (grey bars) *, **, *** depict significant ($p < 0.05, 0.01, 0.005$) attenuation of proliferation rates when T cells were preincubated with TGFβ1-LAP on activating beads. **A)** Transfer of 15 min-incubated bead/T cell conjugates to uncoated plates recapitulated dose-dependent attenuation of T cell proliferation by TGFβ1-LAP on activating beads. These T cells strongly responded to independent anti-CD3/CD28 stimulus on culture plates. This additional proliferative response was inhibited by initial TGFβ1-LAP-presentation in dose-dependent manner; down to ~31% at highest loading of TGFβ1-LAP ($n = 4$). **B)** After 24 h of bead/T cell preincubation T cells responded with additional proliferation to plate-bound anti-CD3/CD28 antibodies. Proliferation on anti-CD3/CD28 plates, however, was restored to nearly the same rates with and without TGFβ1-LAP preincubation on beads; down to ~85% at highest doses ($n = 3$).

difference in proliferation rates whether the 24 h-preincubation was performed with or without TGFβ1-LAP on activating beads. In conclusion, silencing of T cells by TGFβ1-LAP-loaded beads was reverted on longer incubations and thus did not reflect a long-lasting T cell anergic state or T cell death (Additional file 1: Figure S1 documents that T cell viability is unaltered under all experimental conditions).

A range of T cell functions is attenuated when TGFβ1 is presented on activating beads

A pan human T cell cytokine array was used to characterize the range of T cell effector responses which were silenced by TGFβ1-presentation on TCR/CD28-

activating beads (Figure 4). As expected, activating beads alone induced secretion of cytokines IFN-γ, IL-2, IL-4, IL-10, IL-17, TNF-α. Consistent with attenuated TCR-proximal tyrosine phosphorylation and T cell proliferation, bead-bound TGFβ1-LAP held back secretion of these cytokines in dose-dependent manner to nearly baseline levels (Figure 4A). T cell effector functions which were attenuated by TGFβ1-LAP on activating beads included inflammatory responses (TNF-α), T_H1 and T_H2 functions (IFN-γ, IL-4), T_H17 functions (IL-17) and regulatory T cell activities (IL-10). Other cytokines, like IL-8 and IL-16 were secreted by resting T cells and showed little induction following TCR-activation (Figure 4B). Consistently, secretion of these cytokines was not altered by TGFβ1 presentation

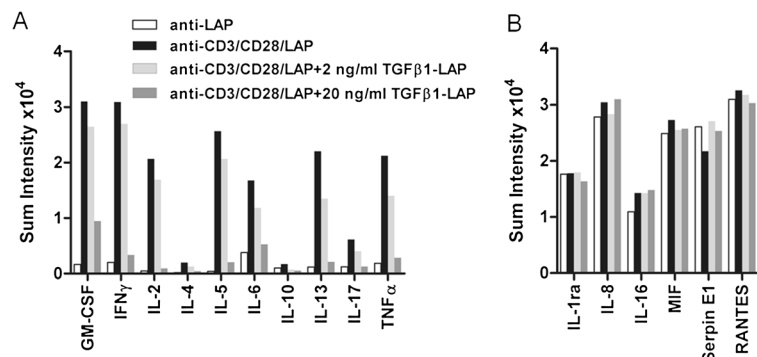


Figure 4 A, B Cytokine array of human pan T cell cytokine secretion-profiles. **A)** TCR/CD28 activating-antibodies on anti-CD3/CD28/LAP beads induced cytokine secretion (black bars) over non-activating control beads (white bars). Loading of TGFβ1-LAP on these TCR/CD28 activating beads suppressed activation-induced secretion of cytokines in a dose-dependent manner (grey bars). **B)** Cytokines which were secreted by resting T cells showed little induction following TCR-activation were not affected by TGFβ1 on TCR/CD28 activating beads. The cytokine array analysis was performed once and is detailed in Additional file 2: Figure S2.

on TCR/CD28 activating beads. Cytokine array analysis was performed once and is fully documented in Additional file 2: Figure S2.

TGF β 1-presentation hinders spreading of T cells over TCR/CD28-activating bead surface

Next, spreading of T cells over anti-CD3/CD28/LAP bead surfaces with and without TGF β 1-LAP loading was characterized by epifluorescence microscopy and statistical analysis (Figure 5). Bead/T cell conjugates, after 45 min incubation at 37°C, were stained with Alexa488 fluorescent anti-actin mAb to visualize the circumference of T cells, and protein G beads were additionally marked with Alexa555 mouse IgG1. Consistent with previous reports, anti-CD3/CD28/LAP antibodies alone induced T cell-spreading over the surface of TCR/CD28-activating beads leading to their engulfment (Figure 5A) and actin-containing structures formed at the edges of the beads while actin was depleted from the central region of bead-cell contact [23,24]. Importantly, loading of TGF β 1-LAP on activating beads significantly inhibited engulfment of TCR/CD28-activating beads (Figure 5B, C). Inhibition of T cell spreading over TCR-activating surfaces was reported to attenuate T cell activation response [23,25].

Conclusions

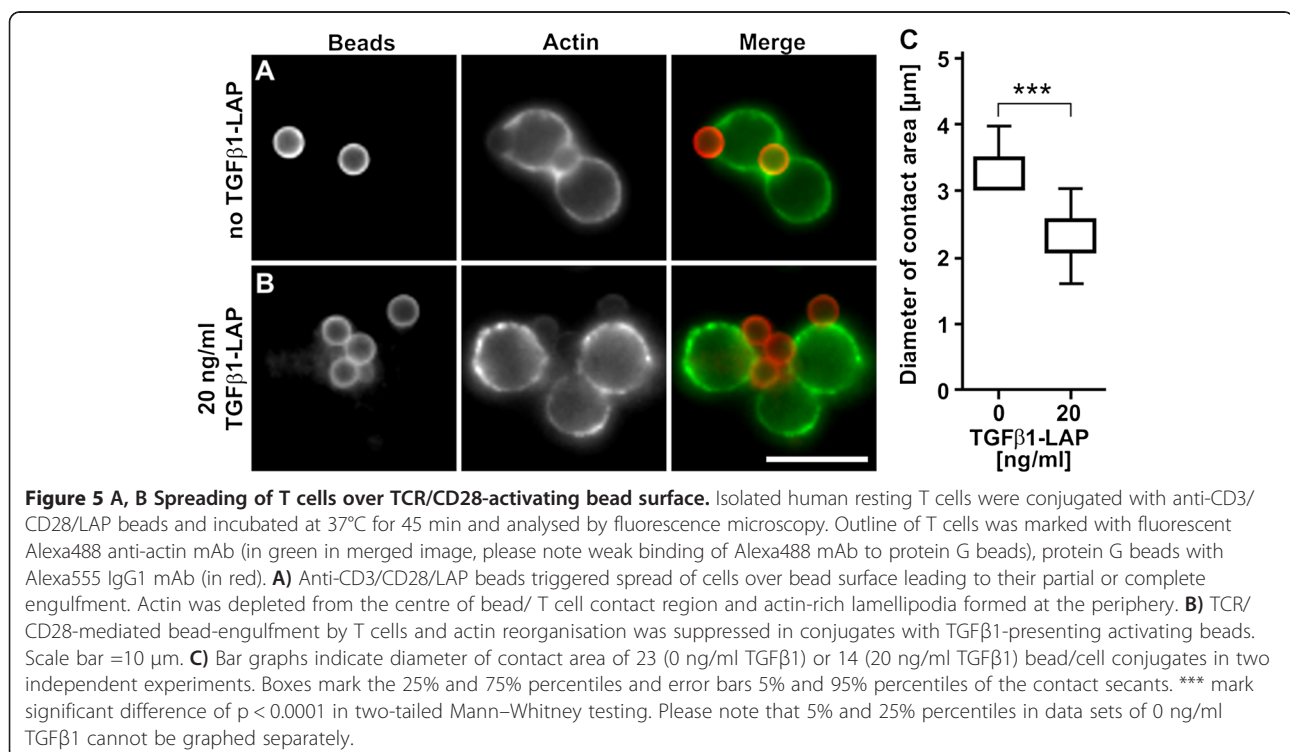
Pleiotropic action and consequent severe side effects preclude use of active TGF β in medical applications

for the treatment of pathological conditions connected to unwanted T cell activity [26]. Our findings suggest a new strategy to circumvent these difficulties by selective targeting of TGF β 1 to TCR activation plasma membrane domains in order to specifically silence T cell responses. Bifunctional biologicals which link functions of TCR activation and TGF β RI and II activation proximally in the plasma membrane promise applications in therapy of disease associated to unbalanced, unwanted T cell activity in autoimmunity, allergy or transplant rejection.

Methods

Materials

Protein G-coated dynabeads were obtained from Novex Life technologies. Pan T cell isolation kit II was from Miltenyi Biotec. HIT3a anti-CD3 mAb and CD28.2 anti-CD28 mAb were from Becton Dickinson. Anti-LAP-TGF β mAb; latent TGF β 1; active TGF β 1 cytokine, cytokine array kit and TGF β cytokine ELISA assay were from R&D Systems. Anti-phospho-PLC γ 1 (Tyr783), anti-phospho-Zap-70 (Tyr319), and anti-phospho-LAT (Tyr191) were obtained from Cell Signaling Technology, anti- β -Actin monoclonal antibody from Sigma. HRP-conjugated donkey anti-mouse IgG or donkey anti-rabbit IgG (Dianova, Hamburg, Germany) were used as secondary antibodies. Ficoll was from Biochrome, [3 H]thymidine from MP Biomedicals, AIM-V culture medium from Invitrogen.



Isolation of resting human T cells

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation of heparinized blood collected from healthy volunteers. Human T cells were further purified by non-T cell depletion using the Pan T cell isolation kit II (Miltenyi Biotec). Purity of isolated pan T cells was $\geq 99\%$. Cells were washed twice and resuspended in serum-free AIM-V culture medium (Invitrogen). The study was approved by the local ethics committee of the Medical Faculty of the Otto-von-Guericke University Magdeburg. Blood donors gave written informed consent.

Ligand-coating of beads, ELISA analysis, and preparation of anti-CD3/CD28 antibody-bound tissue culture plates

0.6 mg of protein G dynabeads were coated with 11 μg of antibody mix; anti-CD3, anti-CD28 and anti-LAP antibodies at 1:5:5 ratios; anti-LAP, anti-CD28 antibodies at 1:1 ratios, or anti-LAP antibodies alone. After wash in 2×1 ml PBS, 0.5% BSA beads were suspended in 800 μl PBS, 0.5% BSA for microscopy, ELISAs, array analysis, and proliferation assays or in 80 μl PBS, 0.5% BSA for preparing Western blot samples. Antibody-coated beads in required minimal volumes were incubated with TGF β 1-LAP to achieve final concentrations of 0, 0.1, 0.2, 0.5, 1, 2, and 5 ng/ 4 μl of bead suspension. Anti-CD3/CD28/LAP beads for Western blot samples were loaded to 20 ng TGF β 1-LAP /4 μl suspension. Bead/TGF β 1-LAP mix was incubated o/n on a rotating wheel at 4°C, suspended in 1 ml of PBS, 0.5% BSA in ice and harvested with a magnet. Pelleted beads were then suspended in PBS, 0.5% BSA, retaining above concentrations of beads and bead-bound TGF β 1-LAP.

ELISA: 3 μg anti-CD3/CD28/LAP protein G beads were loaded with 5 ng latent TGF β 1-LAP, transferred into 100 μl AIMV medium in commercial TGF β -ELISA wells (BD Life Technologies) and incubated for 1 h (grey bars) and 24 h (black bars) at 37°C. 10^5 T cells were present in some wells. Plates were developed according to manufacturer's instruction in quadruplicate wells per condition.

Plastic tissue culture plates were coated with goat anti-mouse secondary antibodies (Dianova) 1:100 in PBS 50 μl /well o/n at 4°C, washed in PBS, and then incubated for 1 h with hybridoma supernatants of anti-CD3 (Okt3) and anti-CD28 (CD28.2) monoclonal antibodies; 1:100 in PBS.

Proliferation-, cytokine secretion-assay, viability assessment and Western blot

10^5 T cells were incubated with 4 μl (2×10^5) of respective ligand-coated beads in round bottom wells of 96 well microtiter plates (Nunc) with 100 μl of serum-free AIM-V medium (six wells per condition). Proliferation was assessed after 3 days by measuring [^3H]thymidine [^3H -TdR] incorporation. [^3H]thymidine was added at 0.2 μCi /well for 3 h.

At the end of the incubation period, cells were harvested and radioisotope incorporation was measured as index of lymphocyte proliferation using a betaplate liquid scintillation counter (MicroBeta, Wallac).

Cytokine secretion profile following 3 days of cell/bead incubation was determined from 10x100 μl AIM-V T cell supernatant per condition and was assayed using commercial cytokine array kit (R&D) according to manufacturer's instruction. Semi-quantitative analysis was performed by determining the background-subtracted sum intensity for each dot on the membrane using Kodak D1 3.6 software. Densitometry values of spots on different membranes were normalized using the results for the positive controls.

Data were analysed, graphed, and subjected to statistical analyses using Graphpad Prism software. *, **, *** depict statistical significance $p < 0.05$, 0.01, 0.005, respectively assessed by one way Anova testing.

Cell viability was determined in parallel cell cultures by propidium iodide (PI) staining. After 4 h, 24 h, and 72 h of incubation with respective activating beads, cells were incubated with PI/RNase staining buffer (BD Pharmingen) for 15 min and percentage of PI-excluding cells was subsequently determined by FACS according to manufacturer's instructions.

For Western blot analyses whole cell lysates were prepared from 10^6 T cells in 20 μl AIM-V medium. T cells were incubated with 4 μl (2×10^6) TCR/CD28 activating beads with and without TGF β 1-LAP coating, incubated at 37°C for 0, 1, 5, 30 min, and lysed in a final volume of 100 μl 1x SDS sample buffer. Proteins, 20 μl sample per lane, were separated in 10% SDS-polyacrylamide gel and transferred to Hybond N membranes by semi-dry blotting. Specific binding was visualized by chemiluminescence (Thermo Scientific, Rockford, USA).

Microscopy

Bead/Cell conjugates were incubated at 37°C for 45 min and adhered to poly-L-lysine coated microscopy slides. After 15 min fixation with 2% PFA cells were permeabilised for 10 min with 0.2% TX100 in PBS, washed in PBS/1% BSA for 30 min. Actin of cells was labelled with an Alexa488 conjugated anti-actin antibody (BD, 558623) and protein G beads were marked with Alexa555-labelled IgG1 mAb (Cell Signaling). Fluorescence images were recorded with a CCD camera (Apogee, KX4) mounted to a fluorescence microscope (Leica, DM IRE2) using Alexa488 (Omega Filters, XF116-2) and Alexa555 (Omega Filters, XF111-2) channel settings at 63x magnification (Leica, Oil, NA 1.40, 506206). Maximal lengths of the secants in 14 (20 ng TGF β 1-LAP) and 23 (0 ng TGF β 1-LAP) at the respective bead/cell contact zones was measured. *** mark significant inhibition ($p < 0.0001$) of bead engulfment by TGF β 1-LAP-loading in two tailed Mann-Whitney testing.

Additional files

Additional file 1: Figure S1. Viability of T cells is unchanged on presentation of TGFβ1-LAP on TCR/CD28-activating beads. Bead T cell conjugates were incubated for indicated times at 37°C and percentage of viable, propidium iodide-excluding cells was determined by FACS analysis.

Additional file 2: Figure S2. Documentation; Cytokine array analysis. A) Arrangement of cytokine-trapping antibodies on array, B) Chemiluminescence film exposure C) Semi-quantitative analysis of cytokine-chemiluminescence signals using software Kodak D1 3.6.

Competing interests

Use of bifunctional biologicals which activate TGFβ and TCR signalling in physical proximity in the plasma membrane in order to silence T cell activation responses is filed as European Patent application 13005586.6 - 1412.

Authors' contributions

TH and DR designed and led the experiments, and TH wrote the paper. KG and LS performed T cell activation assays, and LP microscopic characterisations. BS and TH originally posed the immunological question and DR enabled realisation of the approach. All authors read and approved the final manuscript.

Acknowledgments

This work was performed at the Institute for Molecular and Clinical Immunology, Otto-von-Guericke University Magdeburg, Leipziger Straße 44, D 39120 Magdeburg, Germany. This research was supported by the "Initial Funding" program of the Center of Dynamic Systems (CDS); Biosystems Technology, Magdeburg and by the SFB854 of the Deutsche Forschungsgemeinschaft.

Received: 9 July 2014 Accepted: 3 November 2014

Published online: 08 December 2014

References

1. Massague J: TGFβ signalling in context. *Nat Rev Mol Cell Biol* 2012, **13**:616–630.
2. Li MO, Flavell RA: TGF-β: a master of all T cell trades. *Cell* 2008, **134**:392–404.
3. Tran DQ: TGF-β: the sword, the wand, and the shield of FOXP3(+) regulatory T cells. *J Mol Cell Biol* 2012, **4**:29–37.
4. Marie JC, Liggitt D, Rudensky AY: Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-β receptor. *Immunity* 2006, **25**:441–454.
5. Li MO, Sanjabi S, Flavell RA: Transforming growth factor-β controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 2006, **25**:455–471.
6. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Annunziata N, Doetschman T: Targeted disruption of the mouse transforming growth factor-β 1 gene results in multifocal inflammatory disease. *Nature* 1992, **359**:693–699.
7. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S: Transforming growth factor β 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* 1993, **90**:770–774.
8. Worthington JJ, Fenton TM, Czajkowska BI, Klementowicz JE, Travis MA: Regulation of TGFβ in the immune system: an emerging role for integrins and dendritic cells. *Immunobiology* 2012, **217**:1259–1265.
9. Annes JP, Munger JS, Rifkin DB: Making sense of latent TGFβ activation. *J Cell Sci* 2003, **116**:217–224.
10. Shi M, Zhu J, Wang R, Chen X, Mi L, Walz T, Springer TA: Latent TGF-β structure and activation. *Nature* 2011, **474**:343–349.
11. Kane LP, Lin J, Weiss A: Signal transduction by the TCR for antigen. *Curr Opin Immunol* 2000, **12**:242–249.
12. Riha P, Rudd CE: CD28 co-signaling in the adaptive immune response. *Self Nonself* 2010, **1**:231–240.
13. Nakamura K, Kitani A, Strober W: Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor β. *J Exp Med* 2001, **194**:629–644.
14. Oida T, Weiner HL: TGF-β induces surface LAP expression on murine CD4 T cells independent of Foxp3 induction. *PLoS One* 2010, **5**:e15523.
15. Stockis J, Colau D, Coulie PG, Lucas S: Membrane protein GARP is a receptor for latent TGF-β on the surface of activated human Treg. *Eur J Immunol* 2009, **39**:3315–3322.
16. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, Shevach EM: GARP (LRRC32) is essential for the surface expression of latent TGF-β on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2009, **106**:13445–13450.
17. Baecher-Allan C, Wolf E, Hafler DA: MHC class II expression identifies functionally distinct human regulatory T cells. *J Immunol* 2006, **176**:4622–4631.
18. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, Wang Y, Bernstein X, Huang X, Reichardt LF, Bluestone JA, Sheppard D: Loss of integrin α(v)β8 on dendritic cells causes autoimmunity and colitis in mice. *Nature* 2007, **449**:361–365.
19. Melton AC, Bailey-Bucktrout SL, Travis MA, Fife BT, Bluestone JA, Sheppard D: Expression of αvβ8 integrin on dendritic cells regulates Th17 cell development and experimental autoimmune encephalomyelitis in mice. *J Clin Invest* 2010, **120**:4436–4444.
20. Acharya M, Mukhopadhyay S, Paidassi H, Jamil T, Chow C, Kissler S, Stuart LM, Hynes RO, Lacy-Hulbert A: αv Integrin expression by DCs is required for Th17 cell differentiation and development of experimental autoimmune encephalomyelitis in mice. *J Clin Invest* 2010, **120**:4445–4452.
21. Harder T, Kuhn M: Selective accumulation of raft-associated membrane protein LAT in T cell receptor signaling assemblies. *J Cell Biol* 2000, **151**:199–208.
22. De Crescenzo G, Grothe S, Zwaagstra J, Tsang M, O'Connor-McCourt MD: Real-time monitoring of the interactions of transforming growth factor-β (TGF-β) isoforms with latency-associated protein and the ectodomains of the TGF-β type II and III receptors reveals different kinetic models and stoichiometries of binding. *J Biol Chemistry* 2001, **276**:29632–29643.
23. Bunnell SC, Kapoor V, Triple RP, Zhang W, Samelson LE: Dynamic actin polymerization drives T cell receptor-induced spreading: a role for the signal transduction adaptor LAT. *Immunity* 2001, **14**:315–329.
24. Lowin-Kropf B, Shapiro VS, Weiss A: Cytoskeletal polarization of T cells is regulated by an immunoreceptor tyrosine-based activation motif-dependent mechanism. *J Cell Biol* 1998, **140**:861–871.
25. Campi G, Varma R, Dustin ML: Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med* 2005, **202**:1031–1036.
26. Calabresi PA, Fields NS, Maloni HW, Hanham A, Carlino J, Moore J, Levin MC, Dhib-Jalbut S, Tranquill LR, Austin H, McFarland HF, Racke MK: Phase 1 trial of transforming growth factor β 2 in chronic progressive MS. *Neurology* 1998, **51**:289–292.

doi:10.1186/s12964-014-0074-6

Cite this article as: Harder et al.: Selective targeting of transforming growth factor-β1 into TCR/CD28 signalling plasma membrane domains silences T cell activation. *Cell Communication and Signaling* 2014 **12**:74.

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