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Avidity of human T cell receptor engineered CD4⁺ T cells drives T-helper differentiation fate

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Abstract

The role of the T cell receptor (TCR) in antigen recognition and activation of T lymphocytes is well established. However, how the TCR affects T-helper differentiation/skewing is less well understood, particularly for human CD4⁺ (CD4) T cell subsets. Here we investigate the role of TCR specific antigen avidity in differentiation and maintenance of human Th1, Th2 and Th17 subsets. Two human TCRs, both specific for the same peptide antigen but with different avidities, were cloned and expressed in human CD4 T cells. These TCR engineered cells were then stimulated with specific antigen in unskewed and T-helper skewed conditions. We show that TCR avidity can control the percentage of IL-4 and IFN- γ co-expression in unskewed TCR engineered cells, that effector function can be maintained in a TCR avidity-dependent manner in skewed TCR engineered cells, and that increased TCR avidity can accelerate Th1 skewing of TCR engineered cells.

Keywords

TCR engineered CD4⁺ T cells; TCR avidity; Human T-helper subset differentiation/skewing; Plasticity

1. Introduction

Human naïve CD4⁺ T cells can differentiate into several T-helper (Th) subsets which are categorized primarily by their cytokine and master transcription factor expression [1–4]. Among the most well-characterized of these subset are Th1, Th2 and Th17 cells. Th1 cells express IFN- γ and TNF- α and the master transcription factor T-bet, Th2 cells express IL-4, IL-5 and IL-13 and the master transcription factor GATA3 and Th17 cells express members of the IL-17 cytokine family, IL-21 and IL-22 and the master transcription factor ROR γ t [5–7]. Each subset has effector functions which drive the immune response to clear pathogens. Th1 cells are responsible for cell-mediated immunity to clear viruses and bacteria [8], Th2

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for humoral immunity and helminth regulation [9] and Th17 for a host of intracellular pathogens and fungi clearance [10].

This T-helper subset differentiation or skewing fate can be controlled by both cell extrinsic and intrinsic factors. The extrinsic factors include, but are not limited to, the cytokine milieu, type of antigen/foreign pathogen present, the type of antigen presenting cell (APC) presenting the antigen and the type of co-stimulation [3,11–16]. The intrinsic factors include the TCR signal strength, differential signaling pathway sensitivities, microRNAs and intracellular complement activation [17–22]. Naïve CD4 T cells therefore have to integrate both extrinsic and intrinsic stimuli to determine subset differentiation fate. These stimuli are stratified as per the three signal hypothesis [23,24], which states that TCR recognition of cognate antigen presented in the context of the major histocompatibility complex class II, expressed on the surface of an APC, is the first signal necessary for CD4 T cell activation, proliferation and subsequent differentiation. Signal one is followed by the second signal, which is APC expressed co-stimulatory molecules such as CD80 and CD86 engaging with the CD28 receptors on T cells. This engagement enhances activation and survival of the T cells, prevents anergy and serves as a safeguard for aberrant T cell activation [25,26]. Finally, the third signal is the cytokine milieu, which is largely determined by innate immune cells activated by alarmins and pathogens which trigger pattern recognition receptors [27,28].

Evidence of TCR avidity/functional affinity and thus, signal strength, playing a dominant role in determining T-helper subset fate has been reported. In an *in vivo* study investigating murine T-helper subset determination, the authors observed that TCR signal strength for its cognate antigen predominates the extrinsic factors of APC and cytokine milieu [29]. Another study showed that TCR signal strength affects proximal signaling events that promote Th17 differentiation of cells in mice [30]. Given these findings, and the paucity of information on TCR signal strength in determining human T-helper differentiation, we set out to study the role of TCR avidity in determining and modulating human TCR engineered T-helper fates. We utilized two human TCRs derived from CD4⁺ T cells isolated and cloned from a single Hemophilia A subject [7]. These T cell clones were *HLA-DRB1*01:01* (abbreviated hereafter as “DR1”) restricted and specific for the same peptide epitope in the C2 domain of blood coagulation protein FVIII (residues 2191–2210, abbreviated hereafter as “pC2”) [31]. Furthermore, the two clones were phenotyped as Th2 and Th17/Th1 cells, respectively, based on cytokine and transcription factor expression and they had different avidities for their shared cognate antigen pC2, as measured by proliferation assays [7].

Herein we investigated how TCR avidity for its cognate antigen can modulate or determine the differentiation of human TCR engineered CD4 T cells to Th1, Th2 and Th17 subsets. Avidity was interrogated by varying the concentrations of the cognate antigen, pC2, used to stimulate the TCR engineered cells. Because the two cloned TCRs had different avidities for pC2 at a given concentration, experimental conditions were designed to test effects of this difference on the T-helper phenotypes of the TCR engineered cells. Both naïve and effector memory populations were tested under unskewed, T-helper skewed and T-helper skewing conditions.

2. Materials and methods

2.1. TCR cloning

cDNA was generated from CD4⁺ T cell clones derived from a Hemophilia A subject whose T cells responded to a *DR1*-restricted epitope within pC2 and amplified using a 5' rapid amplification of cDNA ends (RACE) with semi-nested PCR technique as published [32]. PCR primer sequences were as follows: pGI (5'-GI CACCGGGIIGGGIIGGGII-3'); pCa1 (5'-AGTCAGATTTGTTGCTCCAG GC); pCa2 (5'-ATACGCGTTCTCTCAGCTGGTACACGG-3'); pCb1 (5'-TTCACCCACCAGCTCAGCTCC-3'); pCb2 (5'-ATACGCGTAGATCTCTGCTTCTGATGGC-3'). PCR amplified bands were gel-purified and used to transform TOP10 competent *Escherichia coli* cells (Invitrogen) using a TOPO-TA cloning kit as per the manufacturer's instructions (Invitrogen). DNA was isolated from successful transformants, as determined by blue/white screening, and sequenced. Productive sequence reads of the target TCR alpha and beta chains were verified via IMGT (*international ImMunoGeneTics*) and NCBI (National Center for Biotechnology Information) Blast bioinformatic databases. The determined TCR alpha and beta chains were designed as a construct to be expressed as one self-cleaving transcript, using a P2A cis acting hydrolase element sequence inserted at the junction between the end of the alpha and the beginning of the beta chains. The designed construct, including leader sequences and a furin recognition sequence to excise the P2A amino acids left after TCR translation and cleavage, were subcloned into a pRetrox-IRES-ZsGreen vector (Clontech) for retroviral production.

2.2. Retrovirus production and T cell transduction

Ampho293T packaging cells (kind gift from Dr. Brian Schaefer, USUHS) were seeded in 10 mM tissue culture dishes with RPMI 1640 media (Corning Cellgro) supplemented with 10% FBS. They were then transfected via lipofectamine LTX (Invitrogen) as per the manufacturer's instructions, with 7 µg pAmpho envelope plasmid (Clontech), 7 µg pEQ Pam3 (-e) GAG POL plasmid (gift of Dr. Dario Vignali, University of Pittsburgh) and 7 µg of pRETROX-IRES-ZsGreen vector (Clontech) encoding the engineered TCR sequence. Virus particles were collected 48 and 72 h post-transfection, syringe-filtered and stored at -80 °C until transduction.

Sorted non-hemophilic human naïve (CD25⁻ CD127⁺CD45RA⁺) or CCR6⁺ (CD45RA⁻) CD4⁺ T cells were transduced with TCRs encoding or mock retrovirus particles via a RetroNectin (Takara)-mediated spinoculation technique. Briefly, non tissue culture treated 24 well plates (Costar) were coated as per the manufacturer's instructions with RetroNectin at 10 µg/ml and incubated overnight at 4 °C. RetroNectin-coated plates were subsequently blocked for 1 h with 2% BSA and washed in RPMI 1640 media. TCR encoding or mock retroviral particles were added to the RetroNectin plates and centrifuged at 1500g, 37 °C for 90 min. Naïve or CCR6⁺ CD4 T cells were activated for 48–72 h with anti-human CD3 (OKT3 clone-Biolegend) and anti-human-CD28 (28.2 clone-Biolegend) and then added to the viral particle/RetroNectin coated wells, and the plates were centrifuged at 1500g at room temperature for 10 min. Cells were then incubated at 37 °C and expanded in RPMI media with appropriate concentrations of IL-2 (NCI Frederick).

2.3. Tetramer and Vbeta2 staining

PE-conjugated DR1 tetramer loaded with peptide pC2 (kind gift from Dr. Kathleen Pratt, USUHS) was incubated with TCR engineered or mock-transduced non-hemophilia A CD4 T cells for 1 h at 37°C at a final concentration of 5 µg/ml in RPMI 1640 (Corning Cellgro) media supplemented with 10% FBS, 1% Human serum AB (Valley Biomedical), 1% Glutamax (Gibco) and human IL-2 (NCI Frederick) at 200 Units/ml media. Tetramer-positive cells were next co-stained by incubating them for 15 min with biotinylated TCR Vbeta2 on ice. The cells were then washed and stained with APC-conjugated streptavidin (Biolegend).

2.4. T-helper differentiation

Human naïve CD4⁺ T cells (CD45RA⁺ CD127^{hi} CD25⁻) were sorted from healthy non-hemophilic donor PBMCs, seeded at 1×10^6 /ml and activated with plate-bound anti-human CD3 and anti-human CD28 (both from Biolegend) at 5 µg/ml and 2 µg/ml, respectively, under human T-helper differentiating/skewing conditions for 7–9 days. For Th0 (i.e. non-differentiating) conditions, cells were cultured in supplemented RPMI media (see below). For Th1 differentiation, cells received human IL-12 (R&D Systems) at 30 ng/ml and anti human IL-4 (R&D Systems) at 500 ng/ml. For Th2 differentiation, cells received human recombinant IL-4 (R&D Systems) at 50 ng/ml and anti-human IFN-γ (Peprotech) at 2.5 µg/ml. For Th17 differentiation, sorted human CCR6⁺ CD4⁺ T cells (CD45RA⁻ CD127^{hi} CD25⁻) were activated in the presence of IL-23 (Peprotech) at 20 ng/ml, IL-1β (Peprotech) at 10 ng/ml and TGF-β (Peprotech) at 200 pg/ml.

All cells, in the presence of differentiating or non-differentiating conditions, were cultured in RPMI 1640 media (Corning Cellgro) supplemented with 10% FBS, 1% Human serum AB (Valley Biomedical) and 1% Glutamax (Gibco). Human IL-2 (NCI Frederick) was added to the media at 200 Units/ml for Th0, Th1 and Th2 conditions and at 4 Units/ml for Th17 conditions.

To transduce the differentiating T-helper cells, activated cells were added to retronectin- and viral-coated plates after 48 h under the various differentiating conditions. Transduction proceeded as the cells expanded under T-helper differentiating conditions for up to 11 days.

2.5. Proliferation assay

TCR engineered cells were labeled with cell proliferation dye efluor 450 (eBioscience) according to the manufacturer's instructions and were seeded at $0.5\text{--}1.0 \times 10^6$ cells/ml. The cells were incubated at different time points with either specific antigen pC2 (New England Peptide) or non-specific antigens: Factor VIII C2 domain peptide 2218–2237 (New England Peptide), soluble anti-CD3 (Biolegend) or OVA peptide 323–339 (New England Peptide), in the presence of γ-irradiated (6000 rads) autologous or heterologous human HLA-DR1 PBMCs at a 5:1 PBMC to T cell ratio.

2.6. Intracellular staining

TCR engineered cells were incubated with the antigens for various times, washed and rested for up to 72 h and then re-stimulated with $1 \times$ phorbol 12-myristate 13-acetate (PMA),

Ionomycin, brefeldin A and monensin cocktail (eBioscience) for 5–7 h. Cells were harvested and surfaced stained with anti-human CD4 PeCy5 (Biolegend) in FACS buffer (homemade). Fixable viability dye efluor 780 (eBioscience) was added and cells were incubated for 30 min on ice, washed and then incubated in 1× fixation/permeabilization buffer (eBioscience) overnight at 4 °C. After fixation/permeabilization, cells were stained for cytokines and transcriptions factors in 1× permeabilization buffer(eBioscience) overnight at 4 °C. Cytokines were stained using anti-IFN- γ PeCy7, anti-IL-2 BV510, anti-IL-4 PE (all from Biolegend) and anti-IL-17A PerCPCy5.5 (eBioscience). The transcription factors were stained using anti-T-bet PeCy7, anti-GATA3 PE (both Biolegend) and anti-ROR γ t APC (eBioscience). All data were acquired using a BD LSR II flow cytometer and analyzed using Flowjo software (Flowjo, LLC).

3. Results

3.1. TCR cloning and expression

Sequencing of the TCR α and β chains of the human Factor VIII-specific T-cell clone 17195 was described previously [33]. This clone had a Th2 phenotype, was DR1-restricted, and was specific for pC2. A second DR1-restricted, FVIII-pC2-specific clone isolated from the same subject at the same time point in his anti-Factor VIII immune response, 171911, had a Th1/Th17 phenotype. The TCR for this clone was also sequenced. Both TCRs shared the same beta chain allele family and the alpha and beta chains had an 86% amino acid sequence identity (Fig. 1A and B). Retroviral based expression plasmids for both 17195 and 171911 TCRs were designed to make viral particles (Fig. 1C). An empty plasmid (with no TCR sequence inserted) served as a mock control. All expression plasmids encoded for a GFP analogue as a marker for transduction. Using a retroviral transduction protocol, both TCRs were expressed in human naïve and effector memory CD4⁺ T cells from healthy, non-hemophilic subjects. Responsiveness to pC2 was evaluated by proliferation assays. As observed for their respective original clones [7], both 17195 and 171911 TCR engineered T cells proliferated to the specific antigen epitope pC2, although at different rates (Fig. 1D). The proliferation of the TCR engineered cells was specific to pC2, as no proliferation was observed when cells were stimulated with a different FVIII peptide epitope or with an Ovalbumin peptide (Fig. 1E). By measuring expansion of GFP⁺ TCR transduced cells, the 17195 TCR engineered cells showed greater proliferation, which was used as a readout for avidity, at each pC2 concentration tested relative to the 171911 TCR engineered cells (Figs. 2A and S1). This difference in proliferation was not due to different expression levels of the two TCRs as measured by surface anti-TCR Vbeta2 staining (Fig. 2B and C). Evaluation of DR1 tetramer staining patterns and mean fluorescence intensity (MFI) values confirmed that the 17195 TCR engineered cells had a higher avidity for the pC2-loaded tetramer compared to the 171911 TCR engineered cells. The difference in avidity between the TCR engineered cells was especially evident when the cells were tetramer stained seven (7) days post TCR transduction (Fig. 2B). These cells had not been exposed to pC2 and therefore no antigen-specific proliferation had occurred to amplify tetramer binding. Although tetramer binding was increased for both TCR engineered cells after stimulation across a pC2 dose titration curve, the difference in avidity was still observed (Fig. 2C).

3.2. T-helper phenotype and cytokine expression of activated TCR engineered cells

Given the fact that the two TCRs were isolated from T-effector clones having distinct phenotypes, it was of interest to investigate the cytokine expression profiles of the non-hemophilia A CD4 T cells engineered to express these TCRs. Donor naïve CD4 T cells engineered to express both TCRs and stimulated with pC2 produced heterogeneous populations of Th1 (IFN- γ) and Th1/Th2 (IL-4) T-helper phenotypes. The lower avidity engineered cells expressing 171911 TCR produced a greater percentage of Th1/Th2 hybrid cells at all but one stimulatory pC2 concentration compared to the 17195 TCR engineered cells (Fig. 3A). Neither IL-17A nor IL-10 was expressed from the TCR engineered cells after stimulation with pC2 or non-specific stimulation (data not shown). Despite the fact that 17195 and 171911 TCRs were isolated from Th2 and Th17/Th1 clones, respectively, neither of the TCR engineered cells showed a bias or preferential skewing towards the T-helper phenotypes of their parental clones. Rather, the TCRs appeared to function as activation switches or triggers that relayed proliferative signals to the host T cells in a dose dependent manner.

To investigate what regulatory mechanisms may have driven the different frequencies of the Th1/Th2 phenotype between pC2 proliferated 17195 and 171911 TCR engineered cells, intracellular levels of the T-helper transcription factors T-bet, GATA3 and ROR γ t were measured (Figs. 3B and S2). While no marked differences in ROR γ t expression were observed between the two types of TCR engineered cells, differences were observed in the percentages of T-bet and GATA3 double-positive cells (T-bet⁺GATA3⁺). Shifts in the percentage T-bet⁺ GATA3⁺ cells serve as an indicator of loss or gain in Th1 and Th2 phenotypes. There was a decrease in the percentage of T-bet⁺GATA3⁺ and an increase in T-bet single positive (T-bet⁺). 17195 TCR engineered cells following stimulation with higher concentrations of pC2. 171911 TCR engineered cells, in contrast, maintained a steady percentage of T-bet⁺GATA3⁺ cells at these higher concentrations of pC2. These results suggest that the Th1/Th2 hybrid phenotype was regulated at the transcription factor level. Thus TCR avidity can affect the T-helper phenotype of unskewed naïve CD4 T cells.

When the 17195 and 171911 TCRs were expressed in human effector memory CCR6⁺ (CD196) CD4 T cells and stimulated with pC2, a mixed phenotype was observed, consisting of Th17, Th1 and Th17/Th1 hybrid cells. In humans it has been shown that CD4 T cells expressing the chemokine receptor CCR6 preferentially differentiate to Th17 cells [34]. In our flow cytometry sorting protocol using healthy donor peripheral blood, CCR6⁺ cells are seen in the effector memory population of CD4 T cells, while they are absent from naïve (CD45RA⁺) CD4 T cells (Fig. 3C). Accordingly, 17195 and 171911 TCR engineered CCR6⁺ cells exhibited a combination of Th1 (based on IFN- γ expression), Th17 (based on IL-17A expression) and Th17/Th1 hybrid phenotypes when stimulated by either the specific cognate antigen pC2 (Fig. 3D) or by non-specific antigens (data not shown). Higher pC2 concentrations resulted in smaller percentages of Th17 cells with a concomitant increase of Th1 and Th1/Th17 hybrid cells, suggesting that strong TCR stimulation negatively regulates Th17 cells under these conditions.

3.3. T-helper skewed TCR engineered cells effector function modulation following pC2 triggering

The effect of TCR avidity for the cognate antigen on CD4 cells skewed to different T-helper subsets was evaluated next. These experiments tested the extent to which the engineered TCRs modulate CD4 cells that have already been skewed to a specific T-effector phenotype. To start the experiment, healthy donor naïve CD4 T cells were isolated from peripheral blood and skewed to Th1 or Th2 subsets or left unskewed, i.e. Th0. The higher avidity 17195 TCR was then expressed in each skewed subset via retroviral transduction. Transduction alone did not affect the skewing of the cells. TCR transduced cells had T-helper phenotypes that were indistinguishable from untransduced skewed cells (Fig. 4A). Skewed TCR engineered cells were stimulated with either 0.005 µg/ml (low concentration) or 0.05 µg/ml (high concentration) of pC2. These two concentrations were chosen because they exhibited the best differential in proliferation kinetics for 17195 TCR engineered cells.

When co-cultured with irradiated DR1 PBMCs and stimulated with pC2 for 96 h, the proliferated Th1-skewed 17195 TCR engineered cells showed a slight reduction in IFN-γ expression relative to cells stimulated with no pC2 (Fig. 4B). Although observed following stimulation with both low and high concentrations of pC2, the IFN-γ reduction was greater with the high concentration. A loss of viable cells was not responsible for the observed IFN-γ reduction. Surprisingly, this reduction was observed despite an increase in T-bet⁺ cells. The increased percentage of T-bet⁺ cells was reflected in the concomitant decrease of T-bet⁺ GATA3⁺ cells (Fig. 4C). Even with the reduction in IFN-γ the Th1-skewed 17195 TCR engineered preserved their Th1 phenotype following pC2 stimulation. The cells did not acquire a Th0 or Th1/Th2 hybrid phenotype.

Similar to those that were Th1 skewed, Th2-skewed 17195 TCR engineered cells also preserved their phenotype following pC2 stimulation (Fig. 4B). Stimulation with the high concentration of pC2, however, resulted in a smaller percentage of IL-4 expressing cells relative to no pC2 and the low concentration. Despite this reduction in IL-4 expression, the percentage of T-bet⁺ GATA3⁺ Th2-skewed 17195 TCR engineered cells was stable across all pC2 concentrations tested suggesting the loss of IL-4 may be transient (Fig. 4C).

Taken together, these results suggest pre-skewed TCR engineered cells can maintain their T-helper phenotype after antigen specific proliferation. Although greater TCR stimulation via higher concentrations of specific antigen can result in diminutions of Th1 and Th2 signature cytokines, plasticity of the cells is not observed.

3.4. Effector phenotypes of TCR engineered cells following pC2 activation -under T-helper differentiation conditions

After determining how TCR avidity for the cognate antigen could help maintain or attenuate the T-helper phenotype of proliferating, pre-skewed engineered cells, we next tested how this avidity affected T-helper differentiation of the engineered cells. We asked if T-helper differentiation could be accelerated in the presence of cognate antigen and if TCR avidity could promote or impair T-helper fates. Human naïve (CD45RA⁺ CCR6⁻) CD4⁺T cells were transduced with either high-avidity 17195 TCR or low-avidity 171911 TCR and co-

cultured with human irradiated DR1 PBMCs, a high or low concentration of the cognate antigen pC2, under Th0, Th1 or Th2 differentiating conditions. To study Th17 differentiation, human effector memory (CD45RA⁻ CCR6⁺) 17195 and 171911 TCR engineered CD4⁺ T cells were utilized. These TCR engineered cells were co-cultured for 96 and 168 h based on peak cognate antigen proliferation (96 h) and maximal T-helper subset differentiation (168 h). To analyze the 168 h time point, antigen was washed away after 96 h and the cells resuspended under appropriate T-helper differentiation conditions for the remaining 72 h.

After 96 h, the higher-avidity 17195 TCR engineered cells stimulated with both high (0.05 µg/ml) and low (0.005 µg/ml) pC2 concentrations under Th1 differentiating conditions were successfully skewed to Th1 cells, based on IFN-γ expression and lack of IL-4 expressing populations. In contrast, the lower-avidity 171911 TCR engineered cells failed to differentiate to a Th1 phenotype after 96 h and instead had a Th0 signature of IFN-γ⁺ IL-4⁺ double producers (Fig. 5A and B). When Th2 differentiation was assessed for the TCR engineered cells at 96 h, both the 17195 TCR and 171911 TCR engineered cells lacked a Th2 cytokine signature (Fig. 5A and B). This observation was consistent with the phenotype of untransduced naïve human CD45RA⁺ CCR6⁻ cells differentiated under Th2 conditions for 96 h. Similarly, consistent with our Th17 differentiating times for untransduced human CD45RA⁻ CCR6⁺ cells, none of the TCR engineered cell exhibited an optimal Th17 cytokine signature (in terms of IL-17A expression) at 96 h following stimulation with either high or low pC2 concentrations (Fig. 5A and B).

After determining that cognate antigen stimulation did not accelerate differentiation of the TCR engineered cells to Th2 and Th17 effector phenotypes, cells were harvested at the 168-h (7 day) time point after co-culture. As mentioned above, this time point is optimal for T-helper differentiation of untransduced human naïve CD4 T cells to Th1 and Th2 phenotypes, and of effector memory cells to the Th17 phenotype. The 17195 TCR engineered cells cultured under Th1 conditions exhibited mostly IFN-γ⁺ IL-4⁺ double producers by 168 h, a phenotype uncharacteristic of Th1 cells, at the lower concentration of pC2 (Fig. 5A). In contrast, 171911 TCR engineered cells differentiated to Th1 by 168 h at both the high and low pC2 concentrations (Fig. 5B). When Th2 differentiation was assessed at 168 h, 17195 TCR engineered cells stimulated with 0.05 µg/ml of pC2 exhibited a Th2 cytokine signature (Figs. 5A and S3). 171911 TCR engineered cells required a tenfold higher pC2 concentration to differentiate to a Th2 phenotype (Figs. 5B and S3). The Th2 differentiation of TCR engineered cells thus required lower cognate antigen stimulation for the higher-avidity 17195 TCR cells.

Successful Th17 differentiation at 168 h was achieved for the 17195 TCR engineered cells with all tested concentrations of pC2. The 171911 TCR engineered cells had a lower percentage of IL-17A expressing cells compared to mock and 17195 TCR engineered cells when stimulated with pC2 for 168 h. The reduced population of IL-17A expressing cells correlated with increased IL-4 expression amongst the 171911 TCR engineered cells (Fig. 5B). This increase in IL-4 expression was not seen in Th17-skewed 17195 TCR engineered cells or mock transduced cells, and it may have affected Th17 differentiation of the 171911 TCR engineered cells [35]. Interestingly, stimulation with pC2 and non-specific stimulation

with anti-CD3 reduced the percentage of IL-17A expressing cells both of the TCR engineered cells and in mock transduced cells relative to cells cultured with no antigen (Fig. 5C). This corroborates observations from others that strong TCR stimulation down regulates IL-17A expression [36].

In addition to the cytokine data, expression of the master T-helper transcription factors T-bet, GATA3 and ROR γ were measured as markers for T-helper differentiation of the TCR engineered cells. Although it is known that all three of these transcription factors can be expressed simultaneously in unskewed naïve cells [37,38] and, in our hands, effector/memory CD4⁺ T cells, shifts in expression of each transcription factor can be observed following skewing.

Accordingly, a shift to a greater percentage of T-bet⁺ 17195 TCR engineered cells was observed following pC2 stimulation for 96 h under Th1 conditions. There also was an increase in T-bet⁺ GATA3⁺ 17195 TCR engineered cells following stimulation for 168 h under Th2 conditions (Fig. S4). Despite successful Th17 skewing, no increases in ROR γ expression were observed in the proliferated 17195 TCR engineered cells at the pC2 concentrations tested. When these transcription factors were measured in the 171911 TCR engineered cells, a greater percentage of cells expressing T-bet⁺ GATA3⁺ were observed under Th1 and Th2 conditions relative to 17195 TCR engineered cells cultured under matched conditions (Fig. S4). Again, these results suggest that TCR avidity can regulate T-helper transcription factor expression when TCR engineered cells are skewed in the presence of cognate antigen.

4. Discussion

Herein we investigated the role that TCR avidity/functional affinity for cognate antigen plays in driving T-helper phenotypes of human CD4 T cells engineered to express specific TCRs. CD4 T-helper cells play a pivotal role in directing immune responses and ensuring long lasting protection from repeat assaults. As antigen-specific adoptive T cell therapies are an increasingly viable option to control and ameliorate not only cancer but also other disorders [39–42], the ability to manipulate and harness the functional properties of TCR engineered CD4 T cells has readily apparent applications. It is appreciated, for example, that Th2 cells promote robust antibody responses. Therefore, Th2-centered adoptive therapy could help vaccination strategies targeted for humoral immunity. Although both Th1 and Th2 cells have been implicated in promoting effective cytotoxic lymphocyte (CTL) responses against tumors, research suggests that Th1 cells are more efficient at generating the essential anti-tumor CTL memory repertoire [43,44]. Th17 cells also can be used therapeutically to treat certain disorders [45,46]. Thus, development of methods to generate and/or maintain T-helper differentiation of human TCR engineered cells is timely. To the best of our knowledge, this is the first study to investigate antigen-specific TCR avidity and its effects on the T-helper phenotypes of human TCR engineered cells.

We evaluated CD4 T cells transduced with two TCRs that were *HLA-DRI*-restricted to an epitope in a Factor VIII C2 domain peptide (pC2). These TCRs had different avidities for the cognate antigen pC2 as assessed by dose proliferation and tetramer staining. The higher

avidity 17195 TCR engineered cells required 100 fold less pC2 concentration to undergo proliferation above background levels, relative to the lower avidity 171911 TCR engineered cells. Of interest, the pC2-stimulated 171911 TCR engineered cells consistently had a greater percentage of IFN γ /IL-4 double producers relative to 17195 TCR engineered cells. Although based on just two TCRs, this observation suggests that lower avidity of an exogenous TCR for its cognate antigen can steer human T cells to a Th1/Th2 phenotype. These observations also indicate that Th1/Th2 human T-helper cells can exist at certain TCR signaling thresholds, mirroring findings reported in murine models of T-helper cell differentiation *in vivo* and *in vitro* [47]. Reports have also shown that Th1/Th2 cells are stable and may represent a more potent T-helper phenotype during an immune response [48,49].

We also investigated how the TCR avidity of TCR engineered cells affected the maintenance and stability of their pre-skewed T-helper phenotypes. We observed that modulations in their Th1 and Th2 phenotypes were dependent on cognate antigen concentration. For both Th1 and Th2-skewed TCR engineered cells, stimulation with higher pC2 concentrations reduced the number of IFN γ and IL-4 single-expressing cells, respectively. This reduction may be due to a negative regulatory program initiated in the cells after strong TCR stimulation or could have been a transient phenotype [49,50].

In these pre-skewed experiments, it is not surprising that the T-helper phenotype was mostly preserved following pC2 stimulation. Overcoming T-helper skewing and observing plasticity without addition of extrinsic cytokines or other immunomodulatory factors was an unlikely event in these assays. Of note, studies have suggested that *in vitro* and *in vivo* skewing can have disparate effects on epigenetic modifications [51,52]. The epigenetic signature of T-helper skewed cells can influence the degree of plasticity and stability of the cell phenotype [51]. Nonetheless, the present study does confirm that *in vitro* T-helper skewed TCR engineered cells can maintain their phenotype following cognate antigen stimulation.

We also tested whether TCR avidity, and by extension signal strength, predominates both the APC-provided co-stimulation and the cytokine milieu during subset fate decisions. In these experiments, the TCR engineered cells were co-cultured with irradiated PBMCs and stimulated with different concentration of pC2 under T-helper differentiation conditions. High avidity TCR interactions with cognate antigen can accelerate Th1 differentiation relative to differentiation following low-avidity interactions. The high avidity TCR engineered cells required four (4) days, as opposed to the expected seven (7) days, for Th1 differentiation. This observation was of interest because it suggests that TCR avidity can affect the kinetics of T-helper fates. We further observed that Th17 differentiation, as measured by IL-17A expression, is affected by strength of TCR signaling. This observation can be exploited when designing Th17 based adoptive therapies. The choice of a low avidity TCR engineered Th17 cell will be of value if the related immunopathology of IL-17A has to be balanced with its beneficial effects in pathogen clearance.

TCR signal strength is a critical parameter in shaping the CD4 T cell repertoire [53–55]. The ensuing repertoire [56] can contain TCRs with different as well as identical avidities/

functional affinities for a single antigen MHC complex. While TCR avidity seems a plausible candidate for the role of dominant determinant in T-helper differentiation fate, certain reports have shown that TCRs of identical specificities and avidities can still result in different cellular fates [57], and even one TCR can give rise to several fates [58]. Models have been proposed espousing either a probabilistic or a deterministic element governing T-helper subset fate [59]. At the individual cell level, T-helper cytokine expression has been shown to be a stochastic process fitting to a probabilistic model. Yet one could argue that mounting a proper immune response to a life-threatening pathogen is too critical to leave to chance and probability. We hypothesized that there is a deterministic element to T-helper differentiation fate which involves inherent properties of the TCR. The two C2 epitope specific TCRs, having different avidities for their cognate antigen pC2 and engineered into human T cells, allowed us to test this hypothesis.

The higher avidity TCR was isolated from a Th2-typed parental clone and the lower avidity TCR from a Th17/Th1 parental clone [7]. Initially this led us to investigate if these TCRs could bias the host CD4 T cells in which they were expressed to differentiate toward the respective phenotypes of their parental clones. This outcome was not observed. However, conditions which may reveal such a parental clone bias could exist. Because the TCR engineered cells still expressed endogenous TCRs that may have been clustered in the lipid raft signaling synapse with the engineered TCRs, the true *in vivo* inherent properties of the engineered TCRs may not have been fully realized.

Such TCR inherent properties, including but not limited to avidity and affinity for cognate antigen, to direct T-helper subset differentiation have been reported for murine CD4 T regulatory (Tregs) cells, IL-10 secreting cells and Th17 cells [60–63]. In a similar vein, it was shown that TCRs cloned from human and Rhesus macaque viral suppressive CD8 T cells and expressed in species-homologous naïve CD8 T cells rendered those cells suppressive as well. As for human CD4 T cells, one study reported that a single TCR from a naïve CD4 T cell primed *in vitro* could give rise to multiple T-helper subsets, thus suggesting what the authors termed intra-clonal heterogeneity [58]. This observation supports the idea of the TCR serving more as a switch, or trigger, to activate the cell and playing a lesser role in determining T-helper fate. However, this study did not rule out the possibility that TCR inherent properties could direct the subset fate of human naïve CD4 T cells. The deep sequencing data in this study revealed that some TCR sequences were unique to a particular subset, i.e. there was interclonal heterogeneity, while other sequences were shared by at least two and as many as three of the four subsets assayed. While the *in vivo* forces that determine T-helper subset fate are indeed complex, and there is likely more unknown than known, in particular for human T-helper subset fate, current knowledge suggests that TCR inherent properties command an important role in deciding this fate in some cases. Characterization of these cases merits further research.

How might TCR stimulation influence T-helper differentiation? Most studies addressing this question come from murine models. One such study showed that TCR signal strength can be translated into the graded cleavage and thus activation of the proximal signaling paracaspase MALT1 [30]. Targets of MALT1 activation, roquin and regnase-1, were shown to have a direct effect on differentiation of Th17 cells. It has also been shown that the TCR signal

strength can affect MAP kinase signaling substrate JNK2 and the SAP Fyn-mediated signaling pathway, both of which can control Th1 versus Th2 differentiation [5,20]. Further weak signals sensed by the TCR can promote IL-4 and GATA 3 expression, ultimately resulting in a Th2 phenotype [64].

While not studied herein, Tregs are a T-helper subset that plays a vital role in immune homeostasis and balancing the immune response of CD4 effector cells. Recently, our lab has shown that expression of the 17195 TCR in human natural Tregs can activate the suppressive activity of these Tregs in the presence of the cognate antigen pC2, effectively preventing pC2-triggered proliferation of 17195 TCR engineered effector cells [33]. This cognate antigen-specific suppression represents an important development in the treatment of anti-drug antibodies, termed “inhibitors”, which can occur in hemophilia A subjects receiving FVIII replacement therapy. These inhibitory antibodies are class switched and dependent upon CD4 T cell help [65–67]. Currently, experiments where both high and low avidity TCRs are expressed in induced Tregs (iTregs) are planned. These experiments will shed light on how the avidity of T-effector derived TCRs influences the differentiation of naïve human CD4 T cells to iTregs. Furthermore, these experiments will allow us to test how TCR avidity influences the suppressive functions and plasticity of iTregs—two clinically important parameters for Treg adoptive therapies [68].

In summary, we observed that the T-helper effector phenotype of human TCR engineered cells can be influenced by TCR avidity. Unskewed/undifferentiated TCR engineered cells can be driven to produce Th1/Th2 hybrid cells via low-avidity TCR cognate antigen interaction. While pre-skewed TCR engineered cells maintained their phenotypes during cognate antigen proliferation, TCR avidity influenced the percentages of T-helper cytokine expressing cells that proliferated. Finally, the avidity of the TCR engineered cells affected the kinetics and quality of the skewing in the presence of cognate antigen and T-helper differentiation conditions. Further investigations of these phenomena will aid strategies for TCR selection when engineering antigen-specific T-helper cells to produce and maintain defined effector phenotypes for adoptive therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

APC	antigen presenting cells
HLA	human leukocyte antigen

IL	interleukin
IMGT	international immunogenetics
MHC	major histocompatibility complex
PBMCs	peripheral blood mononuclear cells
pC2	FVIII C2 domain peptide 2191–2210
TCR	T cell receptor

References

1. Chizzolini C, Parel Y, Scheja A, Dayer JM. Polarized subsets of human T-helper cells induce distinct patterns of chemokine production by normal and systemic sclerosis dermal fibroblasts. *Arthritis Res Ther*. 2006; 1:R10. [PubMed: 16356198]
2. Hamann D, Baars PA, Hooibrink B, van Lier RW. Heterogeneity of the human CD4+ T-cell population: two distinct CD4+ T-cell subsets characterized by coexpression of CD45RA and CD45RO isoforms. *Blood*. 1996; 9:3513–3521. [PubMed: 8896418]
3. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol*. 2010:445–489. [PubMed: 20192806]
4. Rautajoki KJ, Kylaniemi MK, Raghav SK, Rao K, Lahesmaa R. An insight into molecular mechanisms of human T helper cell differentiation. *Ann Med*. 2008; 5:322–335. [PubMed: 18484344]
5. Yang D, Conze D, Whitmarsh AJ. Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. *Immunity*. 1998:575–585. [PubMed: 9806643]
6. Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res*. 2006:3–10. [PubMed: 16467870]
7. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. Lineages of human T-cell clones, including T helper 17/T helper 1 cells, isolated at different stages of anti-factor VIII immune responses. *Blood*. 2009; 7:1423–1428. [PubMed: 19549986]
8. Romagnani S. T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol*. 2000; 1:9–18. [PubMed: 10923599]
9. Panzer M, Sitte S, Wirth S, Drexler I, Sparwasser T, Voehringer D. Rapid in vivo conversion of effector T cells into Th2 cells during helminth infection. *J Immunol*. 2012; 2:615–623. [PubMed: 22156341]
10. Romagnani S, Maggi E, Liotta F, Cosmi L, Annunziato F. Properties and origin of human Th17 cells. *Mol Immunol*. 2009; 1:3–7. [PubMed: 19193443]
11. Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. *Nat Immunol*. 2001; 2:102–107. [PubMed: 11175801]
12. Bedoui S, Heath WR. Kruppel-ling of IRF4-dependent DCs into two functionally distinct DC subsets. *Immunity*. 2015; 5:785–787. [PubMed: 25992853]
13. Tsai HC, Wu R. Cholera toxin directly enhances IL-17A production from human CD4+ T cells. *J Immunol*. 2013; 8:4095–4102. [PubMed: 24043897]
14. Guo F, Iclozan C, Suh WK, Anasetti C, Yu XZ. CD28 controls differentiation of regulatory T cells from naive CD4 T cells. *J Immunol*. 2008; 4:2285–2291. [PubMed: 18684917]
15. de Wit J, Souwer Y, van Beelen AJ, de Groot R, Muller FJM, Klaasse Bos H, Jorritsma T, Kapsenberg ML, de Jong EC, van Ham SM. CD5 costimulation induces stable Th17 development by promoting IL-23R expression and sustained STAT3 activation. *Blood*. 2011; 23:6107–6114. [PubMed: 21926348]
16. Murray JS. How the MHC selects Th1/Th2 immunity. *Immunol Today*. 1998; 4:157–163. [PubMed: 9577091]

17. Liszewski MK, Kolev M, Le Friec G, Leung M, Bertram PG, Fara AF, Subias M, Pickering MC, Drouet C, Meri S, Arstila TP, Pekkarinen PT, Ma M, Cope A, Reinheckel T, Rodriguez de Cordoba S, Afzali B, Atkinson JP, Kemper C. Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. *Immunity*. 2013; 6:1143–1157. [PubMed: 24315997]
18. Li Z, Wu F, Brant SR, Kwon JH. IL-23 receptor regulation by Let-7f in human CD4+ memory T cells. *J Immunol*. 2011; 11:6182–6190. [PubMed: 21508257]
19. Kylaniemi MK, Kaukonen R, Myllyviita J, Rasool O, Lahesmaa R. The regulation and role of c-FLIP in human Th cell differentiation. *PLoS ONE*. 2014; 7:e102022. [PubMed: 25019384]
20. Cannons JL, Yu LJ, Hill B, Mijares LA, Dombroski D, Nichols KE, Antonellis A, Koretzky GA, Gardner K, Schwartzberg PL. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. *Immunity*. 2004; 5:693–706. [PubMed: 15539155]
21. Kim JS, Sklarz T, Banks LB, Gohil M, Waickman AT, Skuli N, Krock BL, Luo CT, Hu W, Pollizzi KN, Li MO, Rathmell JC, Birnbaum MJ, Powell JD, Jordan MS, Koretzky GA. Natural and inducible TH17 cells are regulated differently by Akt and mTOR pathways. *Nat Immunol*. 2013; 6:611–618. [PubMed: 23644504]
22. Tao X, Constant S, Jorritsma P, Bottomly K. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *J Immunol*. 1997; 12:5956–5963. [PubMed: 9550393]
23. Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. *Science*. 1996; 5271:104–106. [PubMed: 8658175]
24. Goral S. The three-signal hypothesis of lymphocyte activation/targets for immunosuppression. *Dial Transplant*. 2011; 1:14–16.
25. Appleman LJ, Boussiotis VA. T cell anergy and costimulation. *Immunol Rev*. 2003; 161–180. [PubMed: 12670403]
26. Harris NL, Ronchese F. The role of B7 costimulation in T-cell immunity. *Immunol Cell Biol*. 1999; 4:304–311. [PubMed: 10457196]
27. Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK, Mescher MF. Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4+ and CD8+ T Cells. *J Immunol*. 1999; 6:3256–3262. [PubMed: 10092777]
28. Oppenheim JJ, Tewary P, de la Rosa G, Yang D. Alarmins initiate host defense. *Adv Exp Med Biol*. 2007; 185–194. [PubMed: 17713005]
29. van Panhuys N, Klauschen F, Germain RN. T-cell-receptor-dependent signal intensity dominantly controls CD4(+) T cell polarization In Vivo. *Immunity*. 2014; 1:63–74. [PubMed: 24981853]
30. Jeltsch KM, Hu D, Brenner S, Zoller J, Heinz GA, Nagel D, Vogel KU, Rehage N, Warth SC, Edelmann SL, Gloury R, Martin N, Lohs C, Lech M, Stehklein JE, Geerlof A, Kremmer E, Weber A, Anders HJ, Schmitz I, Schmidt-Supprian M, Fu M, Holtmann H, Krappmann D, Ruland J, Kallies A, Heikenwalder M, Heissmeyer V. Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation. *Nat Immunol*. 2014; 11:1079–1089. [PubMed: 25282160]
31. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. HLA-DR-restricted T-cell responses to factor VIII epitopes in a mild haemophilia A family with missense substitution A2201P. *Haemophilia*. 2010; 102:44–55. [PubMed: 20536985]
32. Wälchli S, Løset GÅ, Kumari S, Nergård Johansen J, Yang W, Sandlie I, Olweus J. A practical approach to T-cell receptor cloning and expression. *PLoS ONE*. 2011; 11:e27930. [PubMed: 22132171]
33. Kim YC, Zhang AH, Su Y, Rieder SA, Rossi RJ, Ettinger RA, Pratt KP, Shevach EM, Scott DW. Engineered antigen-specific human regulatory T cells: immunosuppression of FVIII-specific T- and B-cell responses. *Blood*. 2015; 7:1107–1115. [PubMed: 25498909]
34. Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. *J Immunol*. 2008; 1:214–221. [PubMed: 18097022]
35. Cooney LA, Towery K, Endres J, Fox DA. Sensitivity and resistance to regulation by IL-4 during Th17 maturation. *J Immunol*. 2011; 9:4440–4450. [PubMed: 21949021]

36. Purvis HA, Stoop JN, Mann J, Woods S, Kozijn AE, Hambleton S, Robinson JH, Isaacs JD, Anderson AE, Hilken CMU. Low-strength T-cell activation promotes Th17 responses. *Blood*. 2010; 23:4829–4837. [PubMed: 20713963]
37. De Fanis U, Mori F, Kurnat RJ, Lee WK, Bova M, Adkinson NF, Casolaro V. GATA3 up-regulation associated with surface expression of CD294/CRTH2: a unique feature of human Th cells. *Blood*. 2007; 10:4343–4350. [PubMed: 17234745]
38. Fang M, Xie H, Dougan SK, Ploegh H, van Oudenaarden A. stochastic cytokine expression induces mixed T helper cell states. *PLoS Biol*. 2013; 7:e1001618. [PubMed: 23935453]
39. Fry TJ, Mackall CL. T-cell adoptive immunotherapy for acute lymphoblastic leukemia. *Hematol Am Soc Hematol Educ Program*. 2013:348–353.
40. Miyazaki Y, Fujiwara H, Asai H, Ochi F, Ochi T, Azuma T, Ishida T, Okamoto S, Mineno J, Kuzushima K, Shiku H, Yasukawa M. Development of a novel redirected T-cell-based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia. *Blood*. 2013; 24:4894–4901. [PubMed: 23641014]
41. Oh JW, Seroogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, Dekruff RH, Umetsu DT. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J Allergy Clin Immunol*. 2002; 3:460–468. [PubMed: 12209095]
42. Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G, Spratt SK, Surosky RT, Giedlin MA, Nichol G, Holmes MC, Gregory PD, Ando DG, Kalos M, Collman RG, Binder-Scholl G, Plesa G, Hwang WT, Levine BL, June CH. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med*. 2014; 10:901–910. [PubMed: 24597865]
43. Nishimura T, Nakui M, Sato M, Iwakabe K, Kitamura H, Sekimoto M, Ohta A, Koda T, Nishimura S. The critical role of Th1-dominant immunity in tumor immunology. *Cancer Chemother Pharmacol*. 2000:S52–61. [PubMed: 10950149]
44. Koup RA, Douek DC. Vaccine design for CD8 T lymphocyte responses. *Cold Spring Harb Perspect Med*. 2011; 1:a007252. [PubMed: 22229122]
45. Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher DA, Tangye SG, Cook MC. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med*. 2008; 7:1551–1557. [PubMed: 18591410]
46. Monin L, Griffiths KL, Slight S, Lin Y, Rangel-Moreno J, Khader SA. Immune requirements for protective Th17 recall responses to *Mycobacterium tuberculosis* challenge. *Mucosal Immunol*. 2015; 5:1099–1109. [PubMed: 25627812]
47. Peine M, Rausch S, Helmstetter C, Frohlich A, Hegazy AN, Kuhl AA, Greveling CG, Hofer T, Hartmann S, Lohning M. Stable T-bet(+)GATA-3(+) Th1/Th2 hybrid cells arise in vivo, can develop directly from naive precursors, and limit immunopathologic inflammation. *PLoS Biol*. 2013; 8:e1001633. [PubMed: 23976880]
48. Kannanganat S, Ibegbu C, Chennareddi L, Robinson HL, Amara RR. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. *J Virol*. 2007; 16:8468–8476. [PubMed: 17553885]
49. Han Q, Bagheri N, Bradshaw EM, Hafler DA, Lauffenburger DA, Love JC. Polyfunctional responses by human T cells result from sequential release of cytokines. *Proc Natl Acad Sci USA*. 2012; 5:1607–1612. [PubMed: 22160692]
50. Zhu J, Huang H, Guo L, Stonehouse T, Watson CJ, Hu-Li J, Paul WE. Transient inhibition of interleukin 4 signaling by T cell receptor ligation. *J Exp Med*. 2000; 8:1125–1134. [PubMed: 11034602]
51. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol*. 2009; 2:91–105. [PubMed: 19151746]
52. Franciosi F, Lodde V, Goudet G, Duchamp G, Deleuze S, Douet C, Tessaro I, Luciano AM. Changes in histone H4 acetylation during in vivo versus in vitro maturation of equine oocytes. *Mol Hum Reprod*. 2012; 5:243–252. [PubMed: 22155671]
53. Savage PA, Boniface JJ, Davis MM. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity*. 1999; 4:485–492. [PubMed: 10229191]
54. Baldwin KK, Trenchak BP, Altman JD, Davis MM. Negative selection of T cells occurs throughout thymic development. *J Immunol*. 1999; 2:689–698. [PubMed: 10395659]

55. Reza JN, Ritter MA. Positive and negative selection in the thymus and the thymic paradox. *Dev Immunol*. 1998; 3:161–168. [PubMed: 9851356]
56. Warren RL, Freeman JD, Zeng T, Choe G, Munro S, Moore R, Webb JR, Holt RA. Exhaustive T-cell repertoire sequencing of human peripheral blood samples reveals signatures of antigen selection and a directly measured repertoire size of at least 1 million clonotypes. *Genome Res*. 2011; 5:790–797. [PubMed: 21349924]
57. Persaud SP, Parker CR, Lo WL, Weber KS, Allen PM. Intrinsic CD4+ T cell sensitivity and response to a pathogen are set and sustained by avidity for thymic and peripheral complexes of self peptide and MHC. *Nat Immunol*. 2014; 3:266–274. [PubMed: 24487322]
58. Becattini S, Latorre D, Mele F, Foglierini M, De Gregorio C, Cassotta A, Fernandez B, Kelderman S, Schumacher TN, Corti D, Lanzavecchia A, Sallusto F. T cell immunity. Functional heterogeneity of human memory CD4 (+) T cell clones primed by pathogens or vaccines. *Science*. 2015; 6220:400–406. [PubMed: 25477212]
59. Hodgkin PD. A probabilistic view of immunology: drawing parallels with physics. *Immunol Cell Biol*. 2007; 4:295–299. [PubMed: 17453010]
60. Zhang H, Stern JNH, Strominger JL. T cell receptors in an IL-10-secreting amino acid copolymer-specific regulatory T cell line that mediates bystander immunosuppression. *Proc Natl Acad Sci*. 2009; 9:3336–3341. [PubMed: 19204292]
61. Hsieh CS, Zheng Y, Liang Y, Fontenot JD, Rudensky AY. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol*. 2006; 4:401–410. [PubMed: 16532000]
62. Marks BR, Nowyhed HN, Choi JY, Poholek AC, Odegard JM, Flavell RA, Craft J. Thymic self-reactivity selects natural interleukin 17-producing T cells that can regulate peripheral inflammation. *Nat Immunol*. 2009; 10:1125–1132. [PubMed: 19734905]
63. Yang Y, Torchinsky MB, Gobert M, Xiong H, Xu M, Linehan JL, Alonzo F, Ng C, Chen A, Lin X, Szczesnak A, Liao JJ, Torres VJ, Jenkins MK, Lafaille JJ, Littman DR. Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. *Nature*. 2014; 7503:152–156. [PubMed: 24739972]
64. Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol*. 2010; 4:225–235. [PubMed: 20336151]
65. Ragni MV, Bontempo FA, Lewis JH. Disappearance of inhibitor to factor VIII in HIV-infected hemophiliacs with progression to AIDS or severe ARC. *Transfusion*. 1989; 5:447–449. [PubMed: 2499958]
66. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell epitope repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost*. 2003; 8:1777–1784. [PubMed: 12911593]
67. Cromwell C, Aledort LM. FEIBA: a prohemostatic agent. *Semin Thromb Hemost*. 2012; 3:265–267. [PubMed: 22460908]
68. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity*. 2009; 5:656–665. [PubMed: 19464988]

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2015.10.003>.

Fig. 1.

Cloning and antigen specificity of engineered T cell receptors. (A) DNA gel showing amplified 17195 and 171911 human T cell receptor (TCR) alpha (α) and beta (β) chains, as indicated above band lanes, using primers based on determined IMGT sequences. Both TCRs were cloned using a 5' prime RACE and semi-nested PCR technique, as detailed in Materials and Methods. The 500 base pair (bp) band is indicated. (B) IMGT database verified sequences of human Variable (V), Joining (J) and Diversity (D) alleles and Amino Acid (AA) junction and Constant regions of cloned 17195 and 171911 TCR α and β chains. (C) Schematic of pRetro-X ZsGreen retroviral construct used to make virus particles for TCR expression. Highlighted are LTR viral promoters, restriction sites used in the MCS to insert the TCR α and β chain sequences, which are stoichiometrically expressed via the p2A (porcine teschovirus) linker sequence removed after translation by way of a furin recognition sequence (FRS), and the IRES-mediated expression of ZsGreen, which is analogous to GFP. Constructs with no TCR sequences were used for Mock viral particle production. (D) Representative dot plots showing proliferation of 17195 TCR (left) and 171911 TCR (right) engineered cells after a four (4) day stimulation with pC2 at 50 ng/ml in the presence of γ-irradiated human DR1 PBMCs. GFP/ZsGreen expression (Y axis) and pacific blue (e450) proliferation dye (X axis). GFP⁺ proliferation dye⁻ (upper left hand quadrants on plots) percentages for 17195 and 171911 TCR engineered cells stimulated with no exogenous antigen were 32% and 20%, respectively. Experiment was repeated on at least three independent occasions with SEMs not exceeding 5% for each gated quadrant percentile. (E) Histogram overlays showing proliferation of TCR engineered cells (left) and Mock transduced engineered cells (right) after a 4-day stimulation with pC2, non-specific OVA peptide 323–339 or polyclonal soluble anti-human CD3.

Fig. 2.

Antigen-specific proliferation and differences in TCR avidity of engineered cells. (A) Proliferation as measured by expansion of the GFP⁺ population of TCR and mock engineered naïve CD4⁺ T cells after a 4 day stimulation with a dose titration curve of pC2 at concentrations indicated in the presence of γ -irradiated DR1 PBMCs. GFP fold-expansion metric controls to quantify transduction efficiency of each group of engineered cells across experiments by dividing the percentage of day 4 GFP⁺ cells at each pC2 concentration by the percentage of GFP⁺ cells before stimulation (day 0) of the respective engineered cell. Each point represents the mean $\pm$ SEM of at least three independent experiments with cells from >three donors. (B) Representative contour plots of HLA DR1 tetramer (Y axis) and TCR Vbeta2 allele (also known as Vbeta20.1) allele (X axis) staining of TCR and mock engineered cells following transduction and 7-day expansion in the presence of IL-2 without peptide or human DR1 PBMCs. Tetramer is loaded with pC2 as described in Section 2. Experiment was repeated on at least three independent occasions with SEMs not exceeding 5% for each gated quadrant percentile. (C) Representative contour plots of HLA DR1 tetramer (Y axis) and TCR Vbeta2 (also known as Vbeta20.1) allele (X axis) staining of 17195 TCR (top) and 171911 TCR (bottom) engineered cells after a 5-day stimulation with pC2 (concentrations indicated above plots) in the presence of γ -irradiated human DR1 PBMCs. Experiment was repeated on three independent occasions with SEMs not exceeding 5% for each gated quadrant percentile.

Fig. 3.

TCR avidity for cognate antigen can control proportion of IFN-γ[±] IL-4[±] double expression in human TCR engineered unskewed naïve cells while having little affect on TCR engineered effector memory cells. (A) Representative plots of IFN-γ (Y axis) and IL-4 (X axis) intracellular cytokine expression of rested and PMA/Ionomycin restimulated GFP⁺ proliferated 17195 TCR (left) and 171911 TCR (right) unskewed naïve (CD45RA⁺ CCR6⁻) engineered cells after a 4-day stimulation with 0.5 μg/ml of pC2 in the presence of γ

irradiated human DR1 PBMCs. Line graphs show percentage of IFN- γ ⁺, IFN- γ ⁺ IL-4⁺ and IL-4⁺ expressing 17195 TCR (top) and 171911 (bottom) engineered cells following a 4-day stimulation against a dose titration curve of pC2 at concentrations indicated in $\mu\text{g/ml}$. Background cytokine expression of the engineered cells was determined by incubating the cells for 4 days with no antigen (i.e. 0 $\mu\text{g/ml}$ of pC2) in the presence of γ irradiated human DR1 PBMCs. Each point and error bar represent mean $\pm$ SEM of at least three independent experiments with three different donors. (B) Bar graph showing the percentage of T-bet⁺ GATA3⁺ expressing cells among GFP⁺ proliferated 17195 TCR (closed bars) and 171911 TCR (open bars) unskewed naïve engineered cells after a 4-day stimulation with the indicated concentrations of pC2 in the presence of γ -irradiated DR1 PBMCs. Error bars represent mean $\pm$ SEM of at least three independent experiments with three different donors. (C) Representative contour plot showing CD45RA (APC conjugated, Y axis) and CCR6, also known as CD196 (BV421 conjugated, X axis) staining of pre FACS sorted healthy human donor peripheral blood CD4 T cells. Pre sorted peripheral blood was enriched for CD4 T cells and this enriched population was gated on live CD4⁺ CD25^{lo} cells. (D) Representative plots showing IFN- γ (Y axis) and IL-17A (X axis-top) or IL-4 (X axis-bottom) intracellular cytokine expression of rested and PMA/Ionomycin restimulated GFP⁺ proliferated 17195 and 171911 TCR unskewed effector memory (CD45RA⁻ CCR6⁺) engineered cells after a 4-day stimulation with the indicated concentrations ($\mu\text{g/ml}$) of pC2 in the presence of γ -irradiated DR1 PBMCs. Experiment was repeated on three independent occasions with SEMs not exceeding 5% for each gated quadrant percentile. Plots show TCR engineered CCR6⁺ cells from two different donors.

Fig. 4.

TCR avidity can modulate human T-helper skewed TCR engineered cells in terms of IFN- γ and IL-4 expression. (A) Bar graph comparing T-helper cytokine (IFN- γ , IL-4 and IL-17A) expression of untransduced and TCR transduced naïve (sorted CD45RA⁺ CCR6⁻ CD25⁻ used for Th1 and Th2 skewing) and CCR6⁺ (sorted CD45RA⁻ CD25⁻ used for Th17 skewing) CD4⁺ T cells. For untransduced groups, sorted cells were activated with plate bound anti-CD3 and CD28 and skewed under appropriate Th1, Th2 or Th17 T-helper cytokine conditions for a minimum of 7 days, rested and then restimulated with PMA/Ionomycin for 5–7 h. For transduced groups, sorted cells were activated with plate-bound anti-CD3 and CD28 and skewed under appropriate Th1, Th2 or Th17 T-helper cytokine conditions for 48 to 72 h, then transduced and expanded as detailed in Section 2. Error bars represent mean $\pm$ SEM of at least three independent experiments for both untransduced and transduced cells with up to three donors. (B) Graphs showing intracellular IFN- γ , IL-2 and IL-4 expression of GFP⁺ proliferated Th0 (unskewed), Th1 and Th2 skewed 17195 TCR engineered cells after a 4 day stimulation with no antigen or with the indicated concentrations of pC2 in the presence of γ irradiated DR1 PBMCs. (C) Bar graph showing the percentage of T-bet⁺ GATA3⁺ expression among GFP⁺ proliferated Th1 and Th2 skewed 17195 TCR engineered cells after a 4-day stimulation with no antigen or with the indicated concentrations of pC2 in the presence of γ -irradiated DR1 PBMCs.

Fig. 5.

TCR avidity can accelerate Th1 differentiation and inhibit Th17 phenotype of human TCR engineered cells. (A) Graphs of day 4 and day 7 T-helper cytokine expression of GFP⁺ proliferated 17195 TCR engineered cells stimulated with either low-dose pc2 (0.005 μ g/ml for Th1 and Th17 conditions and 0.05 μ g/ml for Th2 conditions), left, or high-dose pc2 (0.05 μ g/ml for Th1 and Th17 conditions and 0.5 μ g/ml for Th2 conditions), right, in the presence of γ -irradiated DR1 PBMCs. (B) Graphs as in A for 171911 TCR engineered cells. (C) Graph of day 7 cytokine expression of 17195 and 171911 TCR engineered cells skewed

under Th17 conditions with either no antigen or soluble anti-CD3 (at 0.05 µg/ml) stimulation in the presence of γ -irradiated DR1 PBMCs.

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