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Human CD4⁺ CD25⁺ CD127^{hi} cells and the Th1/Th2 phenotype

Aditi Narsale^a, Rosita Moya^a, and Joanna Davida Davies^{a,*}

^aSan Diego Biomedical Research Institute, San Diego, CA, U.S.A

Abstract

CD4⁺ T cells that co-express CD25 and CD127 (CD25⁺CD127⁺) make up around 20% of all circulating CD4⁺ memory T cells in healthy people. The clinical significance of these cells is that in children with type 1 diabetes their relative frequency at diagnosis is significantly and directly correlated with rate of disease progression. The purpose of this study was to further characterize the CD25⁺CD127^{hi} cells. We show that they are a mix of Th1 and Th2 cells however, they have a significantly higher relative frequency of pre-committed and committed Th2 cells, and secrete significantly higher levels of Th2-type cytokines than CD25[−] memory T cells. Further, these cells are neither exhausted nor senescent and proliferate to the same extent as CD25[−] memory cells. Thus, CD25⁺CD127^{hi} cells are a highly active subset of memory T cells that might play a role in controlling inflammation via anti-inflammatory Th2-type deviation.

Keywords

T cell subsets; CD25⁺CD127^{hi} cells; cytokines; chemokine receptors; Th1 and Th2

1. Introduction

Substantial effort has been dedicated to understanding the role of the various memory T-cell subsets in the regulation of immune responses. Human memory CD4⁺ and CD8⁺ T cell compartments are made up of central memory (CM, CD45RO⁺ CD45RA[−] CCR7⁺ CD28⁺), effector memory (EM, CD45RO⁺ CD45RA[−] CCR7[−]) [1,2], and regulatory (CD45RA[−] Foxp3^{hi} CD25^{hi} CD127^{low}) cells [3]. Functional memory T-cell subsets, defined by the cytokines they express, can be either pro-inflammatory Th1 (IFN- γ) and Th17 (IL-17, IL-22) cells, or anti-inflammatory Th2 (IL-4, IL-5, IL-13) cells, or regulatory (IL-10, TGF- β) cells [4].

Correspondence: Joanna D. Davies, jdavies@sdbri.org.

Conflict of interest statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement:

All authors have contributed substantially to this work, have approved the manuscript, and have agreed to this submission.

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Studies primarily performed in mouse models have shown that lineage-specific cytokines and transcription factors can both positively influence their own expansion, and negatively regulate the differentiation and expansion of other cell subsets. Thus, Th1 cells are positively induced by IL-12 and IFN- γ but negatively influenced by IL-4, while Th2 cells are positively regulated by IL-4 and negatively regulated by the Th1 cytokine IL-12 [5–10]. In addition, the Th2-type transcription factor GATA-3 [11] plays a critical role in promoting the Th2 response and in inhibiting Th1 differentiation [12–15]. Likewise, both IL-4 and IFN- γ inhibit the development of Th17 cells [16,17] and during an immune response this results in a negative association between Th17 and both Th1 and Th2 responses. This type of immune regulation, or deviation, caused by the polarization towards and away from lineage commitment, can enhance either the pro-inflammatory, or anti-inflammatory immune responses.

We have identified a population of CD4⁺ memory T cells by their co-expression of CD25, the high affinity IL-2 receptor, and a high density of CD127, the IL-7 receptor [18,19]. In children newly diagnosed with type 1 diabetes (T1D) these cells are significantly associated with the length of partial remission, measured using insulin dose adjusted glycated hemoglobin (HbA1c) levels (IDAA1c), with an increase in frequency associated with protection. In diabetic children with a partial remission greater than 9 months, the frequency of these cells is similar to that seen in healthy subjects suggesting that they might play a causal role in protection. Ligation of both CD25 and CD127 with their respective cytokine ligands provides pro-survival signals [20], suggesting that survival of this cell subset is actively maintained. CD25⁺CD127^{hi} cells are neither Foxp3⁺ regulatory cells (Treg), nor Tr1 regulatory cells, both of which have been implicated in protection from disease progression in people with T1D [21–23]. In the current study, we have further characterized the CD25⁺CD127^{hi} cell population and find that it is a subset of memory cells capable of secreting effector cytokines on CD3 stimulation. In addition, CD25⁺CD127^{hi} cells express significantly higher levels of Th2-type cytokines than their CD25[−] memory cell counterparts. The data suggest that CD25⁺CD127^{hi} cells might play a role in controlling pro-inflammatory responses by deviation towards Th2-type and away from pro-inflammatory-type responses.

2. Materials and Methods

2.1 Healthy subject population PBMC

Whole blood from healthy donors aged 19–29 years was obtained from the Normal Blood Donor Program at The Scripps Research Institute (TSRI). Human Subjects protocols and consent forms were reviewed and approved by both TSRI IRB and San Diego Biomedical Research Institute (SDBRI) IRB. Whole blood was collected in heparin and processed within 2 hours. PBMC were isolated using standard methods and either used immediately or frozen in liquid nitrogen, as indicated for each experiment.

2.2 CD4⁺ cell subsets identified by Flow Cytometry

Fluorochromes, vendors, catalog numbers and registry identifiers for all antibodies used in Flow Cytometry experiments, including isotype control antibodies, are as shown in Supplemental Table 1.

2.3 Naïve, memory, central memory, effector memory, Treg, CD25⁻ memory, and CD25⁺CD127^{hi} memory cell identification

Naïve, memory, central memory, and effector memory cells [1,2] were identified using standard published makers as follows: Cells were labeled for CD3 CD4 CD45RA CD45RO CCR7, CD28, and CD62L to distinguish dominantly naïve (CD45RA⁺, CD45RO⁻), memory (CD45RA⁻, CD45RO⁺), central memory (CM, CD45RA⁻, CD45RO⁺, CCR7⁺, CD28⁺, CD62L^{+/-}), and effector memory (EM; CD45RA⁻, CD45RO⁺, CCR7⁻) cells. CD62L expression was used to distinguish memory cells that either can (CD62L⁺) or cannot (CD62L⁻) recirculate via lymph nodes. In addition, regulatory cells were identified by their high expression of CD25 and low expression of CD127 as described previously [24,25] using the BD Biosciences Treg cocktail that includes antibodies specific for CD4, CD127 and CD25 with CD3 and CD45RO. CD25⁺CD127^{hi} and CD25⁻ memory cells were also identified using the BD Biosciences Treg cocktail. Fresh or frozen cells were used to identify the different cell subsets, except when CD62L antibody was used in a panel. Only freshly isolated PBMCs were used to identify the CD62L^{+/-} CM or EM cells. Data were acquired on an LSRFortessa and analyzed using FlowJo version 10 (Ashland, OR). Isotype controls were used in every experiment and for every antigen-specific antibody.

2.4 Identification of Th1 and Th2 CD4⁺ T cell subsets

Either unsorted PBMC or sorted cell subsets, as indicated for each experiment, were washed twice in RPMI (Invitrogen) with 10% human AB serum and rested at 37°C overnight. Cells were resuspended in RPMI with 10% human AB serum, HEPES (Gibco BRL), glutamine, penicillin, streptomycin (Irvine Scientific), and 2-mercaptoethanol (Sigma-Aldrich) and cultured in 24 well plates at a concentration of $1-3 \times 10^6$ cell per ml with 50ng/ml PMA (Sigma) and 1μM Ionomycin (Sigma). 1 μl of Brefeldin A (BD Bioscience) per ml medium was added at the beginning of the culture. After 4 hours cultured cells were washed twice. Th1 and Th2 cells were identified by their intracellular co-expression of either IFN-γ and the transcription factor T-bet [25], or IL-4 and the transcription factor GATA-3 [11], respectively. Pro-inflammatory cytokines IL-2, TNF-α, and IL-17, as well as the Th2-type cytokine IL-10 were also measured by intracellular stain and Flow Cytometry.

2.5 Identification of pre-committed Th1 and Th2 cell subsets in unsorted and unstimulated PBMC

In addition to memory cell subset markers, PBMC were labeled for CXCR5, CXCR3, and CCR4 to identify pre-committed Th1 (CXCR5⁻ CXCR3⁺ CCR4⁻) and pre-committed Th2 (CXCR5⁻ CXCR3⁻ CCR4⁺) [27,28] cells.

2.6 Cell subset purification by sorting

CD25⁻ memory, CD25⁺CD127^{hi} memory, and Treg cells were identified using the BD Biosciences Treg cocktail with antibodies specific for CD3 and CD45RO. The 3 cell populations were sorted on a BD FACSARIA high-speed cell sorter. In some experiments, total CD4⁺ naïve cells (CD45RO⁻ CD45RA⁺) were also sorted from the same donors and analyzed. Gates used to sort cell subsets are shown in each relevant figure.

2.7 Measurement of T cell proliferation and cytokine secretion

Sorted CD4⁺ T cell subsets were labeled with CFSE (Molecular Probes) using the standard published protocol [29] before culture, and incubated in duplicate at 10⁵ cells per well in 48 well plates for 48 and 72 hours with 10 µl per well anti-CD3/CD28 coated beads (BD Biosciences), 10% human serum (Gemini), HEPES (Gibco BRL), glutamine, penicillin, streptomycin (Irvine Scientific), and 2-mercaptoethanol (Sigma-Aldrich) in RPMI (Invitrogen). Different plates were set up for different time points. For cytokine analysis, 100 µl of culture supernatant was collected at 48 hours and concentrations of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α were determined by Flow Cytometry using the Cytometric Bead Array following the manufacturer's instructions (BD Biosciences Human Th1/Th2/Th17 CBA kit and Biolegend LegendPlex Human Th2 panel). For cell proliferation, cells were washed twice after 72 hours of culture and CFSE dilution determined by Flow Cytometry. FCS Express 6 from De Novo Software (Glendale, CA) was used to calculate proliferation index and percent divided cells.

2.8 Statistics

Multiple groups were analyzed using One-Way ANOVA or Two – Way ANOVA. Comparisons between groups were analyzed using Tukey post-hoc. A p value of less than 0.05 is considered statistically significant. The level of statistical significance is indicated on the Figures as * p=0.05–0.01, ** p=0.009–0.001, and *** p=0.0009–0.0001 and ****p<0.0001. All analyses were performed with Graphpad Prism (La Jolla, CA).

3. Results

3.1 CD25⁺CD127^{hi} T cells express Th1, Th2 and Th17 cytokines

The cytokine profile of CD25⁺CD127^{hi} cells was determined by stimulating sorted cells *in vitro* with PMA/ionomycin for 4 hours. The frequency of cells that express Th1, Th17 and Th2 type cytokines, and are committed to either the Th1, or Th2, or Th17 lineage, was determined and compared with CD25⁻ cells and Treg cells sorted at the same time (Fig 1). The gates used to sort these three populations is shown in Fig 1A, and representative examples of intracellular cytokine expression in each cell subset, as well as the identification of Th1, Th2 and Th17 cells, is shown in Fig 1B. As expected the relative frequency of Treg cells that express any of the cytokines tested was low. In contrast, cells within the CD25⁺CD127^{hi} cell population express all cytokines tested (Fig 1C), with over 10% committed to either the Th1 or the Th2 lineage (Fig 1D). Moreover, the frequency of CD25⁺CD127^{hi} cells expressing either Th1- or Th2-type cytokines (Fig 1C) and committed to either the Th1 or the Th2 cell lineage (Fig 1D) is significantly higher than it is for CD25⁻

cells. The frequency of cells expressing Th17 cytokines is also higher in CD25⁺CD127^{hi} cells compared to CD25⁻ cells, but this population makes up only around 2% of total cells. Therefore, for the remainder of the study we will focus on the more dominant Th1 and Th2 cell subsets.

3.2 The CD25⁺CD127^{hi} T cell compartment contains a significantly higher frequency of Th2-type cells than CD25⁻ memory cells

Cytokine expression in T cells after a 4 hour stimulation is routinely seen in memory, but not naïve T cells. As such, the higher relative frequency of cytokine positive and lineage committed cells within the CD25⁺CD127^{hi} population compared to the CD25⁻ population might be explained by the fact that sorted CD25⁻ cells include naïve and memory cells whereas more than 95% of CD25⁺CD127^{hi} cells have a memory cell phenotype [18,19]. To directly compare the cytokine profile of CD25⁺CD127^{hi} cells with the CD25⁻ memory cell compartment, PBMC were labeled for CD3, CD4, CD45RA, CD45RO, CD25 and CD127. CD45RO cells were identified in a plot gated on CD3⁺ CD4⁺ cells (Fig 2A) and the co-expression of CD25 and CD127 (Fig 2B and C) on CD45RO⁺ cells was determined. The CD4⁺ CD45RO⁺ memory cell population is made up of CD25⁺CD127^{hi} cells (blue gate), Treg cells (red gate) and CD25⁻ cells (pink gate). The relative frequency of CD25⁺CD127^{hi} cells within the total CD45RO⁺ memory cell pool is around 20 percent (Fig 2D).

To further characterize the CD25⁺CD127^{hi} cell population, the relative frequency of CM and EM CD25⁺CD127^{hi} cells (Fig 2E) that express CD62L (Fig 2F and G) was compared with CD25⁻ cells. The relative frequency of CD62L⁺ and CD62L⁻ CM and EM cells within the CD25⁻ and CD25⁺ CD127^{hi} memory cell compartments is not different (Fig 2H).

To compare the Th1/Th2 profile of CD25⁺CD127^{hi} memory cells with their CD25⁻ memory cell counterparts, we determined the relative frequency of pre-Th2 (CCR4⁺ CXCR3⁻ CXCR5⁻), pre-Th1 (CXCR3⁺ CCR4⁻ CXCR5⁻), committed Th1 and committed Th2 cells in both cell subsets. We also compared to total memory (CD45RO⁺) and naïve (CD45RA⁺) cells. Representative plots identify pre-committed Th1 and Th2 (Fig 3A–D), committed Th2 (Fig 3E) and committed Th1 (Fig 3F) cells. The relative frequency of pre-committed and committed Th2 cells (Fig 3G and H), but not pre-committed and committed Th1 cells (Fig 3I and J), is significantly higher within CD25⁺CD127^{hi} cells compared to CD25⁻ memory cells. As expected, the relative frequency of pre-committed and committed cells is very low in naïve cells.

In a separate experiment we determined whether the pre-committed (Figure 4A) and committed (Figure 4B) Th2 cells in the CD25⁺CD127^{hi} cell population were either CM or EM, and found that they were evident in both CM and EM compartments. Moreover, the frequency of pre-committed (Fig 4A) and committed (Fig 4B) Th2 cells is higher in both the CM and EM compartments of CD25⁺CD127^{hi} cells compared to CD25⁻ cells. In contrast, there is no significant difference in Th1-type cells between CD25⁺CD127^{hi} and CD25⁻ in either the CM or EM compartments (Fig 4C and D).

3.3 CD25⁺CD127^{hi} cells secrete significantly higher levels of Th2 cytokines than their CD25⁻ memory cell counterparts

Functionally, the relevant measure of a Th1 and Th2 population is in the cytokines that they secrete. To test this, CD25⁺CD127^{hi} and CD25⁻ memory cells, and CD45RA⁺ naïve cells were identified using the strategy shown in Fig 2, sorted, and stimulated with anti-CD3/anti-CD28 antibody-coated beads. The supernatants were harvested after 48 hours of culture and cytokines quantified using the cytokine bead assay. CD25⁺CD127^{hi} cells secrete significantly more IL-4 (Fig 5A), IL-5 (Fig 5B), and IL-10 (Fig 5C), all Th2-type cytokines, than either CD25⁻ memory or naïve cells. The level of Th2-type cytokine IL-13 secreted was low and highly variable in all cultures (Fig 5D). In contrast, CD25⁺CD127^{hi} cells secreted similar levels of pro-inflammatory cytokines, IL-6 (Fig 5E), IL-2 (Fig 5F), TNF- α (Fig 5G) and IFN- γ (Fig 5H) compared to CD25⁻ cells. The data overall suggest a Th2 predisposition in CD25⁺CD127^{hi} cells compared to CD25⁻ memory cells and naïve cells.

3.4 The proliferation state and capacity of CD25⁺CD127^{hi} cells is equivalent to CD25⁻ memory cells

Both ongoing proliferation and proliferative capacity in response to stimulation via CD3 was measured. Ongoing proliferation, measured directly *ex vivo* using Ki67 expression, is not significantly different for CD25⁺CD127^{hi} memory, CD25⁻ memory or naïve cell subsets. However, consistent with previous published work, Ki67 expression in Treg is significantly higher than in either naïve or memory cells (Fig 6A and B and [30,31]). The capacity of sorted CD25⁺CD127^{hi} and CD25⁻ cells (Fig 6C and D) to divide in response to anti-CD3/anti-CD28 antibody stimulation (Fig 6E–H), measured as proliferation index and percent divided cells, was indistinguishable between the two cell populations (Fig 6I–J).

4. Discussion

This study was designed to further characterize CD4⁺ CD25⁺CD127^{hi} cells, a non-Treg subset of memory T cells that we have previously shown to correlate with the rate of disease progression in children newly diagnosed with T1D, with a higher frequency of cells correlating with slower rate of disease progression [18,19]. Our new data show that CD25⁺CD127^{hi} cells are present in healthy people at a relative frequency similar to that in T1D patients with the slowest rate of disease progression. Their presence in healthy people suggests that they might play a role in delaying disease pathogenesis in people with T1D. Our new data show that CD25⁺CD127^{hi} cells make up around 20 percent of all CD4⁺ CD45RO⁺ memory T cells and they are a mixture of CM and EM. Like their CD25⁻ memory T cell counterparts, they divide efficiently in response to stimulation via anti-CD3, and they secrete pro-inflammatory cytokines. However, they differ from other memory T-cell populations in that they also secrete high levels of Th2-type cytokines and contain significantly higher relative frequency of pre-committed and committed Th2 cells. Based on these data, we suggest that the clinical significance of CD4⁺ CD25⁺CD127^{hi} cells might be to reduce inflammation in conditions such as T1D by deviating a pro-inflammatory response to an anti-inflammatory Th2-type response.

Human CD4⁺ CD25^{int} Foxp3⁻ cells with enhanced capacity to secrete cytokines after stimulation via CD3 compared to CD25⁻ cells have been described previously. However, their expression of CD127 and Th2-type cytokines was not reported in that study [32]. The significance of a strong Th2 response is that the Th2- lineage-specific cytokine IL-4 [5–10], and transcription factor GATA-3 [13–15] can negatively influence lineage commitment to the pro-inflammatory Th1-type and Th17 response [7,17]. Such polarization influences by Th2 cells might contribute to the protection against a pathogenic autoimmune Th1-type response if present at the same time and in the same microenvironment. The finding that CD25⁺CD127^{hi} cells have a significantly higher relative frequency of pre-committed Th2 and committed Th2 cells, as well as enhanced capacity to secrete Th2-type cytokines, compared to CD25⁻ cells, strengthens the overall notion that CD25⁺CD127^{hi} cells favor Th2-type responses.

Th1 and Th2 cells are functionally distinct lineages that can exist as CM or EM depending on their differentiation state after antigen exposure [33]. Because the majority of CD25⁺CD127^{hi} cells are CM (Fig. 2) we tested the possibility that CD25⁺CD127^{hi} cells with a Th2 phenotype are found predominantly within the CM compartment (Fig 4). We found that this is not the case and that Th2-type CD25⁺CD127^{hi} cells are distributed across both CM and EM differentiated states. This is also true for pre-committed and committed Th1 cells.

Cell subsets with a Th2 bias have been noted previously and include recent thymic emigrants (RTE) [34] and CD4⁺ CD44^{v.low} peripheral precursor cells [35]. We have ruled out the possibility that CD25⁺CD127^{hi} cells are either RTE or CD44^{v.low} precursor cells because they do not express the naïve cell marker, CD45RA. A Th2 bias has also been described in senescent memory T cells [36,37]. However, CD25⁺CD127^{hi} cells are not senescent because they express CD28 and divide in response to anti-CD3 ab as effectively as other memory T cell subsets. T cell senescence, on the other hand, is characterized by loss of the co-stimulatory molecule CD28 [38] and cell cycle arrest [39]. The CD25⁺CD127^{hi} cell population also shows no features of exhaustion. Thus, T cell exhaustion is accompanied by a decrease in secretion of IL-2 and TNF- α [40–42] and low levels of cell surface CD127 [43–45]. In contrast, CD25⁺CD127^{hi} cells, by definition, express high levels of CD127, and they secrete IL-2 and TNF- α as effectively as other memory T cells.

Our analysis of CD25⁺CD127^{hi} cells shows that more than 75% have a pre-committed Th2 or Th1 phenotype. It has been hypothesized that pre-committed T-cell subsets are able to circulate through inflamed tissue, where, upon encounter with specific antigen, they commit to a specific lineage [46] by upregulating stable expression of the relevant transcription factors, T-bet for Th1 [26], GATA-3 for Th2 [11], ROR γ t for Th17 [47], and Foxp3 for Tregs [48–50]. However, the majority of CD25⁺CD127^{hi} cells express CD197 and/or L-selectin (CD62L), both homing receptors for lymph nodes, where T-cell responses to antigen generally takes place. Overall these data suggest that cells within the CD25⁺CD127^{hi} population have the capacity to recirculate to both inflamed tissue and lymph nodes.

The expression of high levels of the IL-7 receptor, CD127, bodes well for long term survival of CD25⁺CD127^{hi} cells since signaling through both receptors provides pro-survival signals

[51]. CD25⁺CD127^{hi} cells also express a high surface density of CD44 and the CD44 variant CD44v6 [18], receptors for hyaluronan (HA), signaling through which inhibits apoptosis [52–54]. Moreover, CD44 co-stimulation induces CD25 expression and signaling in the presence of low levels of circulating IL-2 [55]. It is possible that the relative frequency of CD25⁺CD127^{hi} cells reflects the level of circulating mediators, IL-2, IL-7 and HA, which are stable in healthy individuals, but altered during states of inflammation. As such, the relative frequency of CD25⁺CD127^{hi} cells might be useful as a measure of immune homeostasis because they are highly sensitive to circulating factors that are modulated during inflammation.

In conclusion, our data might suggest that CD25⁺CD127^{hi} T cells play a role in maintaining a non-inflammatory environment by deviating immune responses away from pro-inflammatory Th1 response by deviating towards an anti-inflammatory Th2-type response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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8. Abbreviations

PBMC	peripheral blood mononuclear cells
IRB	Institutional Review Board
mAb	monoclonal antibody
CM	central memory
EM	effector memory
T1D	type 1 diabetes
Treg	Foxp3 ⁺ regulatory T cell

References

1. Sallusto F, Geginat J, Lanzavecchia A. Central Memory and Effector Memory T C ell Subsets: Function, Generation, and Maintenance. *Annu Rev Immunol.* 2004; 22:745–763. DOI: 10.1146/annurev.immunol.22.012703.104702 [PubMed: 15032595]
2. Rosenblum MD, Way SS, Abbas AK. Regulatory T cell memory. *Nat Rev Immunol.* 2015; 16:90–101. DOI: 10.1038/nri.2015.1 [PubMed: 26688349]
3. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochoyov G, Sakaguchi S. Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity.* 2009; 30:899–911. DOI: 10.1016/j.immuni.2009.03.019 [PubMed: 19464196]
4. Hirahara K, Nakayama T. CD4⁺ T-cell subsets in inflammatory diseases: beyond the T_h 1/T_h 2 paradigm. *Int Immunol.* 2016; 28:163–171. DOI: 10.1093/intimm/dxw006 [PubMed: 26874355]

5. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature*. 1996; 383:787–793. DOI: 10.1038/383787a0 [PubMed: 8893001]
6. O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity*. 1998; 8:275–83. DOI: 10.1016/S1074-7613(00)80533-6 [PubMed: 9529145]
7. Paul WE, Seder RA. Lymphocyte responses and cytokines. *Cell*. 1994; 76:241–51. [accessed May 1, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/7904900>. [PubMed: 7904900]
8. Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science*. 2002; 295:338–42. DOI: 10.1126/science.1065543 [PubMed: 11786644]
9. Oswald IP, Caspar P, Jankovic D, Wynn TA, Pearce EJ, Sher A. IL-12 inhibits Th2 cytokine responses induced by eggs of *Schistosoma mansoni*. *J Immunol*. 1994; 153:1707–13. 0022-1767/94/\$02.00. [PubMed: 7913944]
10. Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen- induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med*. 1995; 182:1527–36. [accessed May 1, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/7595222>. [PubMed: 7595222]
11. Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem*. 1997; 272:21597–603. DOI: 10.1074/JBC.272.34.21597 [PubMed: 9261181]
12. Hwang ES, Szabo SJ, Schwartzberg PL, Glimcher LH. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science*. 2005; 307:430–3. DOI: 10.1126/science.1103336 [PubMed: 15662016]
13. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell*. 1997; 89:587–96. DOI: 10.1016/S0092-8674(00)80240-8 [PubMed: 9160750]
14. 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; 16:3–10. DOI: 10.1038/sj.cr.7310002 [PubMed: 16467870]
15. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, Murphy KM. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity*. 1998; 9:745–55. DOI: 10.1016/S1074-7613(00)80671-8 [PubMed: 9846495]
16. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*. 2005; 6:1133–1141. DOI: 10.1038/ni1261 [PubMed: 16200068]
17. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005; 6:1123–1132. DOI: 10.1038/ni1254 [PubMed: 16200070]
18. Moya R, Robertson HK, Payne D, Narsale A, Koziol J, Davies JD. Type 1 Diabetes TrialNet Study Group JD. A pilot study showing associations between frequency of CD4(+) memory cell subsets at diagnosis and duration of partial remission in type 1 diabetes. *Clin Immunol*. 2016; 166–167:72–80. DOI: 10.1016/j.clim.2016.04.012
19. Narsale A, Moya R, Robertson HK, Davies JD. Type 1 Diabetes TrialNet Study Group JD. Data on correlations between T cell subset frequencies and length of partial remission in type 1 diabetes. *Data Br*. 2016; 8:1348–51. DOI: 10.1016/j.dib.2016.07.059
20. Carrette F, Surh CD. IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. *Semin Immunol*. 2012; 24:209–217. DOI: 10.1016/j.smim.2012.04.010 [PubMed: 22551764]
21. Marek-Trzonkowska N, My liwec M, Siebert J, Trzonkowski P. Clinical application of regulatory T cells in type 1 diabetes. *Pediatr Diabetes*. 2013; 14:322–332. DOI: 10.1111/pedi.12029 [PubMed: 23627860]
22. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, Herold KC, Lares A, Lee MR, Li K, Liu W, Long SA, Masiello LM, Nguyen V, Putnam AL, Rieck M, Sayre PH, Tang

- Q. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med*. 2015; 7:315ra189.doi: 10.1126/scitranslmed.aad4134
23. Yao Y, Vent-Schmidt J, McGeough MD, Wong M, Hoffman HM, Steiner TS, Levings MK. Tr1 Cells, but Not Foxp3⁺ Regulatory T Cells, Suppress NLRP3 Inflammasome Activation via an IL-10–Dependent Mechanism. *J Immunol*. 2015; 195:488–497. DOI: 10.4049/jimmunol.1403225 [PubMed: 26056255]
 24. Liu W, Putnam AL, Xu-yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, de St Groth BF, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J Exp Med*. 2006; 203:1701–1711. DOI: 10.1084/jem.20060772 [PubMed: 16818678]
 25. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, de St Groth BF. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006; 203:1693–1700. DOI: 10.1084/jem.20060468 [PubMed: 16818676]
 26. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 2000; 100:655–69. DOI: 10.1016/S0092-8674(00)80702-3 [PubMed: 10761931]
 27. Rivino L, Messi M, Jarrossay D, Lanzavecchia A, Sallusto F, Geginat J. Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4⁺ central memory T cells. *J Exp Med*. 2004; 200:725–35. DOI: 10.1084/jem.20040774 [PubMed: 15381728]
 28. Kim CH, Rott L, Kunkel EJ, Genovese MC, Andrew DP, Wu L, Butcher EC. Rules of chemokine receptor association with T cell polarization in vivo. *J Clin Invest*. 2001; 108:1331–9. DOI: 10.1172/JCI13543 [PubMed: 11696578]
 29. Quah BJC, Parish CR. The Use of Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) to Monitor Lymphocyte Proliferation. *J Vis Exp*. 2010; doi: 10.3791/2259
 30. Duhén T, Duhén R, Lanzavecchia A, Sallusto F, Campbell DJ. Functionally distinct subsets of human FOXP3⁺ Treg cells that phenotypically mirror effector Th cells. *Blood*. 2012; 119:4430–40. DOI: 10.1182/blood-2011-11-392324 [PubMed: 22438251]
 31. Booth NJ, McQuaid AJ, Sobande T, Kissane S, Agius E, Jackson SE, Salmon M, Falciani F, Yong K, Rustin MH, Akbar AN, Vukmanovic-Stejić M. Different Proliferative Potential and Migratory Characteristics of Human CD4⁺ Regulatory T Cells That Express either CD45RA or CD45RO. *J Immunol*. 2010; 184 [accessed September 12, 2017] <http://www.jimmunol.org/content/184/8/4317.long>.
 32. Triplett TA, Curti BD, Bonafede PR, Miller WL, Walker EB, Weinberg AD. Defining a functionally distinct subset of human memory CD4⁺ T cells that are CD25^{POS} and FOXP3^{NEG}. *Eur J Immunol*. 2012; 42:1893–905. DOI: 10.1002/eji.201242444 [PubMed: 22585674]
 33. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol*. 2014; 14:24–35. DOI: 10.1038/nri3567 [PubMed: 24336101]
 34. Hendricks DW, Fink PJ. Recent thymic emigrants are biased against the T-helper type 1 and toward the T-helper type 2 effector lineage. *Blood*. 2011; 117:1239–49. DOI: 10.1182/blood-2010-07-299263 [PubMed: 21048154]
 35. Zhao C, Marrero I, Narsale A, Moya R, Davies JD. CD4(+) CD44(v.low) cells are unique peripheral precursors that are distinct from recent thymic emigrants and stem cell-like memory cells. *Cell Immunol*. 2015; 296:106–14. DOI: 10.1016/j.cellimm.2015.04.002 [PubMed: 25910642]
 36. Stacy S, Krolick KA, Infante AJ, Kraig E. Immunological memory and late onset autoimmunity. *Mech Ageing Dev*. 2002; 123:975–85. [accessed July 25, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/12044946>. [PubMed: 12044946]
 37. Hsu H-C, Mountz JD. Origin of late-onset autoimmune disease. *Immunol Allergy Clin North Am*. 2003; 23:65–82. vi. [accessed July 25, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/12645879>. [PubMed: 12645879]

38. Boucher N, Dufeu-Duchesne T, Vicaut E, Farge D, Effros RB, Schächter F. CD28 expression in T cell aging and human longevity. *Exp Gerontol*. 1998; 33:267–82. [accessed July 25, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/9615924>. [PubMed: 9615924]
39. Effros RB. Role of T lymphocyte replicative senescence in vaccine efficacy. *Vaccine*. 2007; 25:599–604. DOI: 10.1016/j.vaccine.2006.08.032 [PubMed: 17014937]
40. Fuller MJ, Khanolkar A, Tebo AE, Zajac AJ. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. *J Immunol*. 2004; 172:4204–14. [accessed July 25, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/15034033>.
41. Fuller MJ, Zajac AJ. Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol*. 2003; 170:477–86. [accessed July 25, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/12496434>. [PubMed: 12496434]
42. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol*. 2003; 77:4911–27. [accessed July 25, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/12663797>. [PubMed: 12663797]
43. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A*. 2004; 101:16004–9. DOI: 10.1073/pnas.0407192101 [PubMed: 15505208]
44. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, Subramaniam S, Blattman JN, Barber DL, Ahmed R. Molecular Signature of CD8+ T Cell Exhaustion during Chronic Viral Infection. *Immunity*. 2007; 27:670–684. DOI: 10.1016/j.immuni.2007.09.006 [PubMed: 17950003]
45. Zhou S, Ou R, Huang L, Price GE, Moskophidis D. Differential tissue-specific regulation of antiviral CD8+ T-cell immune responses during chronic viral infection. *J Virol*. 2004; 78:3578–600. [accessed July 25, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/15016881>. [PubMed: 15016881]
46. Ley K. The second touch hypothesis: T cell activation, homing and polarization. *F1000Research*. 2014; doi: 10.12688/f1000research.3-37.v1
47. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. 2006; 126:1121–33. DOI: 10.1016/j.cell.2006.07.035 [PubMed: 16990136]
48. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor Foxp3. *Immunity*. 2005; 22:329–341. DOI: 10.1016/j.immuni.2005.01.016 [PubMed: 15780990]
49. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, Rudensky AY. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*. 2007; 445:771–775. DOI: 10.1038/nature05543 [PubMed: 17220874]
50. Lin W, Haribhai D, Relland LM, Truong N, Carlson MR, Williams CB, Chatila TA. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol*. 2007; 8:359–368. DOI: 10.1038/ni1445 [PubMed: 17273171]
51. Carrette F, Surh CD. IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. *Semin Immunol*. 2012; 24:209–17. DOI: 10.1016/j.smim.2012.04.010 [PubMed: 22551764]
52. Bollyky PL, Lord JD, Masewicz SA, Evanko SP, Buckner JH, Wight TN, Nepom GT. Cutting edge: high molecular weight hyaluronan promotes the suppressive effects of CD4+CD25+ regulatory T cells. *J Immunol*. 2007; 179:744–7. [accessed June 26, 2016] <http://www.ncbi.nlm.nih.gov/pubmed/17617562>. [PubMed: 17617562]
53. Borland G, Ross JA, Guy K. Forms and functions of CD44. *Immunology*. 1998; 93:139–48. [accessed July 25, 2017] <http://www.ncbi.nlm.nih.gov/pubmed/9616361>. [PubMed: 9616361]
54. Rajasagi M, Vitacolonna M, Benjak B, Marhaba R, Zöller M. CD44 promotes progenitor homing into the thymus and T cell maturation. *J Leukoc Biol*. 2009; 85:251–61. DOI: 10.1189/jlb.0608389 [PubMed: 18955544]

55. Bollyky PL, Falk BA, Long SA, Preisinger A, Braun KR, Wu RP, Evanko SP, Buckner JH, Wight TN, Nepom GT. CD44 costimulation promotes FoxP3+ regulatory T cell persistence and function via production of IL-2, IL-10, and TGF-beta. *J Immunol.* 2009; 183:2232–41. DOI: 10.4049/jimmunol.0900191 [PubMed: 19635906]

Highlights

- CD25⁺ CD127^{hi} T cells expresses Th1, Th2 and Th17 cytokines
- The CD25⁺ CD127^{hi} T cell pool contains more Th2-type cells than CD25⁻ memory cells
- CD25⁺ CD127^{hi} cells secrete higher levels of Th2 cytokines than CD25⁻ memory cells
- The proliferation capacity of CD25⁺ CD127^{hi} cells is equivalent to CD25⁻ memory cells
- CD25⁺ CD127^{hi} cells are neither exhausted nor senescent

Fig. 1. CD25⁺CD127^{hi} T cells express Th1, Th2 and Th17 cytokines

A) The dot plot is gated on CD3⁺ CD4⁺ cells and shows the gates used to sort CD25⁻ (orange), CD25⁺ CD127^{hi} (red) and Treg (blue) cells from PBMC of healthy adult subjects. B) Representative histograms and dot plots depicting the expression of cytokines (IL-2, IFN- γ , TNF- α , IL-17, IL-4, IL-10) and transcription factors (T-bet, GATA-3, ROR γ t) expressed intracellularly by CD25⁺CD127^{hi} cells (red histogram), CD25⁻ cells (orange histogram) and Tregs (blue histograms). C) Sorted CD25⁻ (closed bars), CD25⁺ CD127^{hi} (open bars) and Treg (hatched bars) from PBMC of healthy adult subjects (n=3 in 3 separate experiments) were stimulated with PMA and ionomycin for 4 hrs. D) The relative frequency of each cell subset that co-expresses either T-bet and IFN- γ , or GATA-3 and IL-4, or ROR γ t and IL-17 (n = 4). Data are analyzed by One Way ANOVA with Tukey post-hoc. A p value <0.05 is considered significant. Significance between cell subsets determined using ** p= 0.009–0.001, *** p= 0.0009–0.0001 and **** p<0.0001.

Fig. 2. CD25⁺CD127^{hi} cells are a mix of central memory and effector memory cells
 CD25⁺CD127^{hi} cells makeup around 20% of total CD4⁺ memory T cells and are evident both within the CM (CD197⁺CD28⁺) and EM (CD197⁻CD28⁺) compartments of the CD45RO⁺ memory cell compartment. A) PBMC from 19–29-year-old healthy males (n=9) are gated on CD3⁺CD4⁺. Total memory cells are identified as CD45RO⁺RA⁻ (black box) and naïve as CD45RA⁺RO⁻ (green box). B) Plot gated on CD45RO⁺ cells from panel A shows the strategy used to identify CD25⁺CD127^{hi} cells (blue box), Treg cells (red box) and CD25⁻ memory cells (pink box). C) An overlay dot plot gated on CD45RO⁺ cells from panel A, labeled with either isotype controls for anti-CD127 and anti-CD25 (blue) or CD127- and CD25-specific antibodies (red) used for selecting the quadrants shown in panel B. D) The stacked histogram shows the frequency of CD45RO⁺ memory cells that are either CD25⁺CD127^{hi} (blue), or Treg cells (red), or CD25⁻ memory cells (pink). Data are mean ±

SD (n = 9). E) A representative plot showing the co-expression of CD197 and CD28 on CD25⁺CD127^{hi} cells used to identify CM (CD197⁺ CD28⁺) and EM (CD197⁻). F) and G) are representative plots showing the expression of CD62L on CM (F) and EM (G) cells. H) Stacked histograms showing the relative frequency of CD62L^{+/+} CM and EM cells within the CD25⁻ and CD25⁺CD127^{hi} memory cell subsets. Data are expressed as mean $\pm$ SD (n = 3).

Fig. 3. CD25⁺CD127^{hi} cells are biased towards a Th2 phenotype

Plots A–D are gated on CD4⁺ CD45RO⁺ CXCR5⁻ cells and show the expression of CXCR3 and CCR4 on either CD25⁻ (A), CD25⁺ CD127^{hi} (B), CD45RO⁺ total memory (C), or CD45RA⁺ naïve cells (D) to identify pre-committed Th1 and Th2 cells. A representative example of committed Th2 cells (E) and Th1 cells (F) in CD4⁺ CD45RO⁺ CD25⁺ CD127^{hi} cells is shown by the co-expression of IL-4 and GATA-3 for Th2 and IFN-γ and T-bet for Th1. The relative frequency of pre-committed Th2 (G), committed Th2 (H), pre-committed Th1 (I), and committed Th1 (J) in CD25⁻ cells, CD25⁺CD127^{hi} cells, total memory and naïve cells was determined in PBMC from healthy young adults (n = 9). Data shown are mean ± SEM analyzed using One-Way ANOVA with Tukey post-hoc. A p value <0.05 is considered significant. Significance between cell subsets determined using * p= 0.05 – 0.01 ** p= 0.009 – 0.001 and **** p<0.0001.

Fig. 4. The higher frequency of Th2-type cells in CD25⁺CD127^{hi} cell compared to CD25⁻ memory cells is evident in CM and EM

PBMC were labeled for CD3 CD4 CD45RA CD25 CD127, CD197, and either CXCR5, CXCR3 and CCR4, or IFN- γ and T-bet, or IL-4 and GATA-3. The relative frequency of either pre-committed Th2 (A), committed Th2 (B), pre-committed Th1 (C) or committed Th1 (D) cells with either a CM or EM phenotype in either CD25⁻ memory (closed bars) or CD25⁺ CD127^{hi} (open bars) compartments was determined. The relative cell frequency is shown as mean $\pm$ SD (n = 3). A Two – Way ANOVA was used to analyze the dataset to determine the relative frequency of Th1- and Th2-type cells within CM and EM CD25⁻ and CD25⁺CD127^{hi} cells.

Fig. 5. CD25⁺CD127^{hi} cells secrete significantly more Th2-type cytokines IL-4, IL-5 and IL-10 than other memory and naïve cells

CD25⁺CD127^{hi} and CD25⁻ memory cells were identified in PBMC from healthy adults using the gates described in Figure 2, sorted and stimulated with anti-CD3/CD28-coated beads for 48hrs. Sorted naïve cells (CD4⁺ CD45RA⁺) were used as an additional control cell population. The levels of secreted IL-4 (A, n = 6), IL-5 (B, n = 6), IL-10 (C, n = 3), IL-13 (D, n = 3), IL-6 (E, n = 4), IL-2 (F, n = 4), TNF-α (G, n = 6) and IFN-γ (H, n = 4) in culture supernatants were determined. Data shown are mean ± SEM analyzed using One-Way ANOVA with Tukey post- hoc. A p value <0.05 is considered significant. Significance between cell subsets is determined using * p = 0.05-0.01 and ****p<0.0001.

Fig. 6. The proliferation state and capacity of CD25⁺CD127^{hi} cells is equivalent to CD25⁻ memory cells

PBMC from 3 healthy donors were labeled to identify CD25⁻ and CD25⁺CD127^{hi} memory cells, total memory cells and naïve cells and the relative frequency of each cell population that expresses Ki67 determined (A). Isotype control expression for Ki67 on Treg cells is shown in (B). Cell subset expression of Ki67 in panel A was compared using One-Way ANOVA with Tukey post-hoc. In a separate experiment CD25⁻ and CD25⁺CD127^{hi} cells were sorted using gates shown in C) and D). Panel C is gated on CD3⁺CD4⁺ cells. Panel D is gated on CD45RO⁺ cells (black box) and shows the gates used to sort either CD25⁻ cells (pink box), or CD25⁺CD127^{hi} cells (blue box). Both sorted populations were CFSE-labeled,

and cultured with anti-CD3 ab plus anti-CD28 ab coated beads for 72 hours and proliferation of CD25⁻ (E) and CD25⁺CD127^{hi} (F) cells measured using CFSE dilution. Unstimulated CFSE controls at 72 hours are shown for CD25⁻ (G) and CD25⁺CD127^{hi} (H) cells. Proliferation index (PI, I) and percent divided cells (J) was determined after 72hr of stimulation for each cell subset using the FCS Express 6 software (n = 4). In each panel data are shown as mean $\pm$ SEM.