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CD4⁺ CD44^{v.low} cells are unique peripheral precursors that are distinct from recent thymic emigrants and stem cell-like memory cells

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Abstract

CD4⁺ CD44^{v.low} cells are peripheral precursor T cells that inhibit lymphopenia by generating a large CD4⁺ T cell pool containing balanced numbers of naïve, memory, and regulatory Foxp3⁺ cells with a diverse TCR repertoire. Recent thymic emigrants (RTE) and stem cell-like memory T cells (T_{SCM}) can also replenish a T cell pool. In this study we formally test whether CD44^{v.low} cells are the same population as RTE and T_{SCM}. Our data show that, in contrast to RTE, CD44^{v.low} cells express high levels of CD45RB and low levels of CD24. Moreover, CD44^{v.low} cells isolated from mice devoid of RTE retain their capacity to repopulate lymphopenic mice with naïve and memory cells and Foxp3⁺ Tregs. In addition, CD44^{v.low} cells do not express IL-2R β , Sca-1, and CXCR3, the phenotypic hallmarks of T_{SCM}. Overall, these data demonstrate that CD44^{v.low} cells are neither RTE nor T_{SCM}.

Keywords

Recent thymic emigrants (RTE); T cell subsets; precursor cells; immune homeostasis; cytokines; stem cell-like memory cells (T_{SCM})

1. Introduction

The size of the T cell compartment and the balance between different T cell subset numbers remains constant by export of new cells from the thymus and by homeostatic mechanisms to

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Authors' contributions

Chunfang Zhao, Idania Marrero and Joanna Davies designed the experiments. The experiments were performed by Chunfang Zhao, Idania Marrero, Aditi Narsale, and Rosita Moya and analyzed by Chunfang Zhao, Aditi Narsale and Joanna Davies. Joanna Davies performed statistical analysis and wrote the manuscript.

Conflict of Interest

The authors declare they have no conflicts of interest.

maintain the peripheral T cell pool. The cells most recently exported from the thymus are given the descriptive name, recent thymic emigrants (RTE). RTE are a distinct transitional T cell subset [1] that seed the peripheral naïve T cell compartment [2-3]. RTE mature in the periphery and fully transition to become mature naïve T cells within 3 weeks of export [2]. In human, thymic export plays a less significant role in replenishing the peripheral immune cell population than it does in rodents, and instead naïve cell proliferation plays a more dominant role in maintaining peripheral naïve cell numbers [4]. In lymphopenic mice, proliferation of naïve cells replenishes a CD4⁺ T cell pool with a predominantly memory, rather than naïve phenotype [5–7]. The precursor cells for both central and effector memory cells are Stem cell-like memory T cells (T_{SCM}), T_{SCM} have a mixed naïve and memory-like phenotype in mice [8-9] and humans [10-11].

CD4⁺ CD44^{v.low} cells are precursor cells for both CD4⁺ naïve and memory cells with a diverse TCR repertoire. They also generate Foxp3⁺ regulatory T cells. CD4⁺ CD44^{v.low} cells express a high density of CD62L and constitute the 2–5% of total naïve CD4⁺ CD44^{low} cells that express the lowest density of CD44 [12], confirming their naïve phenotype [13–15]. Thus, the naïve CD4⁺ CD44^{low} cell population is made up of CD44^{v.low} and CD44 intermediate (CD44^{int}) cells, which we refer to here as precursor cells and naïve cells, respectively.

CD44^{v.low} cells differ from RTE and T_{SCM} in several respects. Unlike RTE they express a high density of CD45RB whereas a low density of CD45RB is one of the hallmark identifiers of RTE [2]. Moreover, whereas T_{SCM} secrete more IL-2 than naïve cells after in vitro stimulation with anti-CD3 and anti-CD28 [10], CD44^{v.low} cells secrete significantly less IL-2 than do naïve cells under these conditions [12]. CD44^{v.low} precursors also differ from naïve cells in their capacity to replenish components within the T cell pool. Thus, CD44^{v.low} cells generate a significantly larger CD4⁺ T cell pool with balanced numbers of naïve, memory, and Foxp3⁺ cells and a diverse TCR repertoire in lymphopenic hosts, while naïve cells generate a dominantly memory phenotype population with significantly fewer Foxp3⁺ cells and a less diverse TCR repertoire [12]. CD44^{v.low} also differ from naïve cells in that they have the capacity to inhibit cachexia, a profound wasting syndrome for which there is no cure or treatment [12]. However, there are also similarities between CD44^{v.low} cells and RTE, T_{SCM}, and naïve cells in their capacity to replenish the naïve and memory cell compartments. While redundancy in mechanisms to maintain such an important function as immune homeostasis might be expected, further studies to determine how CD44^{v.low} cells contribute to an additional mechanism are warranted.

Ideally a precursor T cell would generate a T cell pool with the capacity to develop the many cell lineages that are required to respond effectively to immune challenge. In euthymic mice, a competent naïve CD4⁺ T cell pool can generate Th1 (IFN- γ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17, IL-22), Th22 (IL-22, TNF- α) and Foxp3⁺ regulatory (IL-10 and TGF- β) cells [16]. However, during lymphopenia-induced proliferation (LIP) naïve cells differentiate to generate a T cell pool with a pro-inflammatory cytokine profile [17]. Under the same lymphopenic conditions, a CD4⁺ precursor T cell would be required to generate a T cell pool that is not pro-inflammatory but instead maintains the capacity to secrete all lineage cytokines after cell activation.

In this study we show that peripheral CD44^{v.low} precursor cells are not RTE, neither are they T_{SCM} cells. Further characterization of the cells generated by CD44^{v.low} cells shows that they secrete significantly more Th2-type cytokines IL-4 and IL-6, significantly less Th17/Th22-type cytokines IL-17A and IL-22, but equivalent Th1-type cytokines IFN- γ and TNF- α compared to cells generated by their naïve cell counterparts. Moreover, the Foxp3⁺ cells generated by CD44^{v.low} precursor cells have regulatory function. The capacity of CD44^{v.low} cells to promote a balance cytokine profile might explain their ability to inhibit cachexia.

2. Materials and Methods

2.1. Mice

Euthymic and thymectomized CBySmn.CB17-Prkdc^{scid}/J (CB17.SCID), BALB/cByJ (BALB/c), and C57BL/6 mice were purchased from The Jackson Laboratory. Foxp3^{EGFP} mice on the BALB/c background were a generous gift from Dr. Talal Chatila, University of California, Los Angeles, and were bred at the Torrey Pines Institute for Molecular Studies vivarium. Mice were maintained under specific pathogen-free conditions and were used between 6 and 12 weeks of age. All protocols were approved by the Institutional Animal Care and Use Committee of the Torrey Pines Institute for Molecular Studies and conducted according to institutional guidelines.

2.2. CD4⁺ CD44^{v.low} and CD4⁺ CD44^{int} T cell subset purification and adoptive transfer

Purification of CD4⁺ subsets was as previously described [12]. In brief, splenocytes were isolated from either BALB/c or Foxp3^{EGFP} mice and enriched for CD4⁺ cells by negative selection using a MACS CD4⁺ T cell isolation kit (Miltenyi Biotech) or EasySep mouse CD4⁺ T cell enrichment kit (STEMCELL Technologies). Enriched (70%–85%) CD4⁺ cells were labeled with allophycocyanin-anti-CD4 (RM4-5) and PE-anti-CD44 (IM7) monoclonal antibodies (mAbs), and CD4⁺ CD44^{v.low} and CD4⁺ CD44^{int} cell subsets were purified by sorting on a FACSaria (Becton Dickinson). Supplemental Figure 1 shows the pre- and post-sort profile of CD4⁺ CD44^{v.low} and CD4⁺ CD44^{int} cell subsets including the gates used to sort these populations. For completion it also shows CD44^{hi} cells. Thus, a histogram gated on CD4⁺ T cells shows density of CD44 expression as three peaks. The lowest of the three peaks contains the CD44^{v.low} cells. The highest peak contains predominantly the memory population and the middle peak predominantly the naïve population. CD4⁺ CD44^{v.low} cells represent 5-6% of all CD4⁺ T cells. To purify CD4⁺ CD44^{v.low} cells the CD4⁺ T cells that express the 3% lowest density of CD44 were sorted. Sorted CD4⁺ CD44^{int} cells were the 30% of CD4⁺ cells spanning the CD44 intermediate peak. This strategy significantly reduces the risk of contamination of CD44^{v.low} cells with CD44^{int} cells. The purity of sorted cells was always greater than 99%. For experiments with sorted Foxp3⁺ cells, all cells were sorted from Foxp3^{EGFP} mice. For transfer, sorted cells were washed once in PBS and 1.0×10^5 cells in 100 μ l PBS were injected intravenously (iv) into CB17.SCID recipient.

2.3. Phenotypic analysis by Flow Cytometry

Single-cell suspensions of spleen, pooled lymph node (LN; cervical, inguinal, brachial, para-aortic), and thymus were stained with combinations of the following mAbs: allophycocyanin-conjugated anti-CD4 (RM4-5), FITC-conjugated anti-CD8 α (53-6.7), anti-

CD24 (M1/69), anti-CD44 (IM7), PE-conjugated anti-CD44 (IM7), anti-IL-4 (11B11), anti-IL-6 (MP5-20F3), anti-IL-17A (TC11-18H10), anti-TNF- α (MP6-XT22), anti-CD62L (MEL-14), anti-CD45RB (16A), (all from BD Biosciences), PE-Cy7-conjugated anti-Sca-1 (D7), PerCP/Cy5.5-conjugated anti-CD122 (IL-2R β , TM-b1), BV421-conjugated anti-CXCR3 (CXCR3-173) (all from BioLegend), FITC-anti-FoxP3⁺ (FJK-16s) (all from eBioscience). Data were analyzed using FlowJo version 8.8.6 (Tree Star).

Data were acquired on either an LSRFortessa (Fig.1), or a FACSCalibur (Fig.2, Fig.3, and Fig.4). Fresh single color control samples were set up for each experiment. Aliquots of cells were stained with either anti-CD3 or anti-CD4 conjugated to either FITC, or PE, or PerCP-Cy5.5, or PE-Cy7, or APC, or BV421 depending on the fluorochromes used in each experiment. Single color control samples collected on the FACSCalibur were saved and compensation was performed during analysis using FlowJo software. Single color control samples acquired on the LSRFortessa were used for compensation before acquisition of the experimental samples.

Isotype controls were used in every experiment and for every antigen-specific antibody. Both the isotype and the fluorochrome conjugated to the isotype control were matched for each antigen-specific antibody used. In each case, the concentration of fluorochrome-conjugated isotype control was the same as the concentration of the antigen-specific antibody that it was controlling for.

2.4. In vitro cytokine measurements

Enriched or sorted CD4⁺ T cell subsets were incubated in triplicate at the concentration indicated for each experiment in 96-well plates and stimulated for 2 days with 1 μ g/ml plate-bound anti-CD3 mAb (145-2C11), and 1 μ g/ml soluble anti-CD28 (37.51) mAb (BD Biosciences). Cells were incubated in RPMI (Invitrogen) with 5% fetal bovine serum (Intergen), HEPES (Gibco BRL), glutamine, penicillin, streptomycin (Irvine Scientific), and 2-mercaptoethanol (Sigma-Aldrich). For cytokine analysis, 100 [μ l] of culture supernatant was collected from each well and concentrations of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ , and TNF- α were determined by Flow Cytometry using the Th1/Th2/Th17 Cytometric Bead Array following the manufacturer's instructions (BD Biosciences). IL-22 and TGF- β were measured using ELISA kits according to the manufacturer's instructions (eBioscience). For intracellular cytokine staining, for the last four hours of culture, 1 [μ l] of BD GolgiPlug per ml medium was added to each culture and swirled gently to mix thoroughly. Cultured cells were washed twice and labeled with anti-CD4 mAb. Intracellular IL-4, IL-10, IL-17A, TNF- α , IL-2, IL-6, IFN- γ , and IL-22 content was determined by Flow Cytometry using cytokine-specific antibodies according to the manufacturer's instructions (BD Biosciences).

2.5. In vitro suppression assay

CD4⁺ CD44^{low} cells sorted from BALB/c Foxp3^{EGFP} mice were infused into CB17.SCID mice. Three weeks later the expanded cells were isolated and Foxp3⁺ cells were sorted by expression of GFP. Control Foxp3⁺ Tregs were sorted directly from BALB/c Foxp3^{EGFP} splenocytes. Foxp3⁺ cells were titrated into an MLR consisting of CD4⁺ CD25⁻ cells

enriched from BALB/c spleen as responders and irradiated (3000 rads) C57BL/6 splenocytes as stimulators, at 2×10^5 and 1×10^5 cells per well, respectively. Cultures were incubated for 4 days and harvested following a 16 h pulse with [^3H]-thymidine.

2.6. Single joint T cell receptor rearrangement excision circle (sjTREC) quantification in sorted T cell subsets

$\text{CD4}^+ \text{CD44}^{\text{v.low}}$, $\text{CD4}^+ \text{CD44}^{\text{int}}$ and $\text{CD4}^+ \text{CD44}^{\text{hi}}$ cells were enriched for CD4^+ cells as described above and then sorted using gates described in Supplemental Figure 1. Single positive $\text{CD4}^+ \text{CD3}^+$ thymocytes were sorted directly from total thymocytes preparations. sjTREC were quantified as described previously [18]. Briefly, DNA was extracted from sorted cells by treatment with proteinaseK for 1 hour at 56°C with continuous agitation of the tubes. The DNA was quantified using the NanoDrop Lite Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and amplified with forward sjTREC primer: 5'- CAT TGC CTT TGA ACC AAG CTG -3', reverse sjTREC primer: 5'- TTA TGC ACA GGG TGC AGG TG -3', and detected with probe: 5'- /56-FAM/CA GGG CAG G/ZEN/T TTT TGT AAA GGT GCT CAC TT/3IABkFQ/ -3' (Integrated DNA Technologies, Custom made Oligos, Coralville, Iowa, USA). sjTREC standards (a generous gift from Dr. Gregory D. Sempowski, Duke University, NC, USA) were run simultaneously with the experimental samples to quantify TREC.

2.7. Statistical analysis

Data were analyzed using the unpaired Student t-test, or the Mann–Whitney test, as indicated in each Figure legend. 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 *** $p=0.0009-0.0001$.

3. Results

3.1. $\text{CD4}^+ \text{CD44}^{\text{v.low}}$ cells are phenotypically distinct from RTE and T_{SCM}

To test whether $\text{CD44}^{\text{v.low}}$ cells have the same phenotype as RTE, splenocytes, thymocytes (and LN cells, data not shown) from untreated BALB/c mice were co-stained for CD4 and CD44 as well as either CD24 or CD45RB, markers that distinguish RTE from mature naïve T cells [2]. CD44 subsets are identified as described in Fig.1a. RTE are contained within the CD44^{low} population that expresses a high density of CD24 and a low density of CD45RB (boxed area, Fig.1b) whereas naïve cells express a low density of CD24 and a high density of CD45RB [2]. The expression of CD44 is not different in RTE and naïve cells that are no longer RTE [2]. In contrast to RTE, we show that $\text{CD44}^{\text{v.low}}$ cells express a low density of CD24 and high density of CD45RB, similar to that seen on naïve CD4^+ T cells (Fig.1c). Moreover, whereas RTE and CD4^+ single positive thymocytes have overlapping CD24 and CD45RB expression [2], $\text{CD44}^{\text{v.low}}$ cells express a lower density of CD24 and higher density of CD45RB than do thymocytes. These data show that $\text{CD44}^{\text{v.low}}$ cells are phenotypically distinct from RTE.

Like naïve cells T_{SCM} express a low density of CD44 [15, 19] and a high density of CD62L [13-15], but they also express CXCR3, IL-2R β , and Sca-1, markers generally associated

with memory phenotype cells [8-11]. We have previously shown that CD44^{v.low} cells also express a high density of CD62L in addition to other markers for naïve phenotype cells [20] suggesting that they are a subset of the naïve CD4⁺ T cell compartment. As expected, all three markers are expressed at detectable levels on a subset of CD44^{hi} memory phenotype cells. They are also expressed on a very small subset of naïve CD4⁺ CD44^{int} cells (Fig.1d), similar to that seen previously for murine CD8⁺ T_{SCM} [8]. Our new data show that the expression of CXCR3, IL-2R β , and Sca-1 on CD44^{v.low} cells is very low and not significantly different from that on CD4⁺ CD44^{int} cells. These data indicate that CD44^{v.low} cells are not T_{SCM}.

3.2. CD4⁺ CD44^{v.low} cells are present in spleens of mice six weeks after thymectomy

Within three weeks of thymic export RTE transition from RTE to mature naïve cells [2]. Therefore, if very low expression of CD44 is a marker for RTE CD44^{v.low} cells should be absent from the spleen and lymph nodes of mice that were thymectomized at least three weeks previously. To test this, untreated mice were thymectomized, allowed to rest for six weeks, and tested for the presence of CD44^{v.low} cells in spleen and lymph nodes by Flow Cytometry. Consistent with the loss of RTE, CD44^{low} cells in thymectomized mice have a higher density of CD24 (Fig.2a) and a lower density of CD45RB (Fig.2b). In contrast, our data show that CD44^{v.low} cells are present in euthymic mice (Fig.2c) and mice that were thymectomized six weeks previously (Fig.2d). However, they are significantly reduced in relative frequency (Fig.2e) and number (Fig.2f) after thymectomy. The number of CD44^{int} and CD44^{hi} cells is also reduced after thymectomy (Fig.2f) but the reduction in CD44^{hi} cells is less pronounced. The change in CD44^{int} and CD44^{hi} number results in an increase in relative frequency of CD44^{hi} cells with a concomitant decrease in relative frequency of CD44^{int} cells (Fig.2e). The presence of CD4⁺ cells that express a very low density of CD44 six weeks after thymectomy is consistent with the hypothesis that CD44^{v.low} cells are not RTE.

3.3. CD44^{v.low} cells from mice that are devoid of RTE show CD44^{v.low} precursor cell activity

To determine whether the CD44^{v.low} cells present in thymectomized mice are functional, they were purified from splenocytes by sorting and 1×10^5 of the sorted cells were transferred into SCID mouse recipients. Three weeks later their capacity to repopulate the CD4⁺ naïve, memory, and Foxp3⁺ T cell pool in spleens and lymph nodes was determined by Flow Cytometry. Additional groups of SCID mice were injected with either an equal number of CD44^{v.low} cells sorted from euthymic mice, or CD44^{int} cells sorted from either thymectomized or euthymic mice. The number of total CD4⁺ cells, CD44^{int} and CD44^{hi} cells recovered from spleens and lymph nodes of mice that received CD44^{v.low} cells from thymectomized donors was equivalent to the number recovered from mice injected with CD44^{v.low} cells from euthymic mice, and significantly greater than from mice that received CD44^{int} cells (Fig.3a). Moreover, CD44^{v.low} cells from thymectomized and euthymic mice are equally efficient in generating Foxp3⁺ cells (Fig.3b). The capacity of CD44^{v.low} cells isolated from mice that are devoid of RTE to replenish naïve, memory, and Foxp3⁺ cells confirms that CD44^{v.low} cells are not RTE.

CD24 plays a critical role in optimal T cell proliferation in lymphopenic hosts [21]. Our data show that a significantly greater percent of CD4⁺ cells recovered from mice injected with either CD44^{v.low} or CD44^{int} cells, whether from euthymic or thymectomized donors, express CD24 than CD4⁺ cells recovered from untreated BALB/c mice (Fig.3c).

3.4. CD44^{v.low}-derived cells are biased towards generating cells with a Th2-type phenotype

Our data thus far indicate that CD44^{v.low} cells are neither RTE nor T_{SCM} highlighting the potential importance in having multiple cell subsets and mechanisms to maintain immune homeostasis. A functional CD4⁺ precursor T cell would be required to generate a CD4⁺ T cell pool with a balanced cytokine profile as well as a balanced naïve, memory, and Foxp3⁺ regulatory cell population. To test this, enriched CD4⁺ cells from SCID mice previously injected with CD44^{v.low} cells were stimulated in vitro with anti-CD3 and anti-CD28 under non-polarizing conditions. The cytokines secreted were measured and compared with CD4 cells enriched from either SCID mice injected previously with CD44^{int} cells or from untreated BALB/c mice stimulated at the same time and under the same conditions. The T cell pool generated by CD44^{int} naïve cells is expected to have a pro-inflammatory cytokine profile [17], while the cytokine expression by cells from untreated BALB/c mice is expected to be more quiescent than either of the other two cell subsets. CD4⁺ cells derived from CD44^{v.low} cells secreted significantly more of the Th2-type cytokines IL-4, IL-6, and IL-10, and less IL-2 and Th17-type cytokines IL-17A and IL-22 than cells derived from CD44^{int} cells. Secretion of the Th1-type cytokines IFN- γ , and TNF- α was not different between groups (Fig.4a). The levels of IL-10, IL-2, IFN- γ , and TNF- α were not greater in cells from SCID mice compared to untreated BALB/c mice possibly suggesting that cells expressing these cytokines are not perturbed as a result of LIP. These data show that the CD4⁺ T cell pool generated by CD44^{v.low} cells is significantly more bias towards a Th2 cytokine profile than that generated by CD44^{int} cells.

When the cultured CD4⁺ cells were tested for cytokine expression by intracellular stain we found that the relative frequency of IL-4⁺, and IL-6⁺ cells derived from CD44^{v.low} cells was significantly greater than from CD44^{int} cells, and the relative frequency of IL-17⁺ and TNF- α ⁺ significantly lower (Fig.4b). No differences were detected in expression of IL-10, TGF- β , IL-22, IL-2, and IFN- γ between the groups (data not shown). These data confirm that CD44^{v.low}-derived cells have a bias towards a Th2 profile and away from a pro-inflammatory Th17 profile.

CD4⁺ cells from mice that received either CD44^{v.low} or CD44^{int} cells secrete significantly more of most cytokines than do CD4⁺ cells from BALB/c mice even though none of the groups of mice were primed. Fig.4c shows that the CD4⁺ T cells from the SCID mice are activated as shown by their decreased expression of CD62L. We suggest that their increase in cytokine expression and secretion might be the result of activation.

3.5. Foxp3⁺ cells generated by CD44^{v.low} cells have regulatory function

To determine if CD44^{v.low} cell-derived Foxp3⁺ cells are functional, we performed a standard in vitro suppression assay. Foxp3⁺ cells were sorted from ten CB17.SCID mice reconstituted with Foxp3-CD44^{v.low} cells or, for controls, directly from BALB/c Foxp3^{EGFP}

mice. Control Foxp3⁺ cells sorted from BALB/c mice (Tregs) completely inhibited the MLR when used at a ratio of responders (BALB/c CD4⁺ CD25⁻) to stimulators (irradiated C57BL/6 splenocytes) of 1:1, and substantially inhibited at a ratio of 1:0.25 (Fig.5a). Strikingly, CD44^{v.low} cell-derived Foxp3⁺ cells also strongly suppressed the MLR, even at a ratio of 1:0.25 (Fig.5b), confirming that CD44^{v.low} cell-derived Foxp3⁺ cells are functional Tregs.

3.6. CD44^{v.low} precursor cells contain the same number of TREC as CD4 single positive thymocytes

T cell receptor (TCR) rearrangement excision circles (TREC) are non-replicative DNA byproducts of TCR rearrangement. Thymocytes generate TREC as the TCR is rearranged. Until the cells export the thymus and divide, TREC content remains high. The naïve cell population contains high levels of TREC because most of the cells have not divided and therefore contain TREC. In contrast, the memory T cell population, including T_{SCM}, has low levels of TREC [10, 22]. Because CD44^{v.low} cells are precursors of the naïve CD4⁺ T cell compartment, we predicted that they would have high levels of TREC. To test this, CD44^{v.low} precursor cells were sorted from untreated BALB/c spleens and TREC levels measured. TREC content was compared with resting naïve (CD44^{low} CD62L^{hi}), memory (CD44^{hi} CD62L^{low}) cells, and single positive CD4⁺ thymocytes. TREC content in CD44^{v.low} cells is similar to that seen in thymocytes and significantly higher than in memory cells (Fig.6). These data add to the evidence that CD44^{v.low} cells are not T_{SCM}.

4. Discussion

In this study we have directly tested whether CD4⁺ CD44^{v.low} peripheral precursor cells are either RTE, or T_{SCM}. Our data clearly show that they are not but instead represent an additional cell subset capable of repopulating the CD4⁺ T cell compartment in lymphopenic mice. Further analysis of the CD44^{v.low} cell subset shows that they generate a CD4⁺ T cell pool with an overall cytokine profile that is significantly more Th2-type and less Th17-type than that generated by the CD44^{int} naïve cell compartment, and a functional Foxp3⁺ regulatory cell (Treg) compartment. The result is a cytokine profile that is significantly less inflammatory than that normally associated with LIP.

T_{SCM} express the memory cell markers CXCR3, IL-2R β , and Sca-1 [8-11]. However, they are distinguished from the remaining memory T cells by their co-expression of naïve cell markers including CD44^{low}. T_{SCM} generate both central and effector memory T cells. Like T_{SCM}, CD44^{v.low} cells express naïve cell markers and generate memory phenotype cells. However, CD44^{v.low} cells also generate naïve cells, do not express CXCR3, IL-2R β , and Sca-1, and contain equivalent numbers of TREC as CD4 single positive thymocytes. These data clearly indicate that they are not T_{SCM}.

RTE show several similarities with CD44^{v.low} cells. They generate naïve phenotype cells and they express a low density of CD44. However, CD44^{v.low} and RTE are also different in many respects: CD44^{v.low} cells express a low density of CD24 and a high density of CD45RB, whereas a high density of CD24 and low density of CD45RB is indicative of RTE. Moreover, the remaining CD44^{v.low} cells sorted from mice devoid of RTE by

thymectomy six weeks previously retain their capacity to generate naïve, memory, and Foxp3⁺ Treg cells. These data indicate that CD44^{v.low} cells are distinct from RTE. The significant loss of CD44^{v.low} cells in spleens of mice six weeks after thymectomy suggests that they are continuously exported from the thymus. This loss might be the result of either poor survival of CD44^{v.low} cells or their maturation to naïve phenotype cells. We favor the latter possibility not only because of their known propensity to mature to other cell subsets, but also because CD44^{v.low} cells show enhanced expression of survival factors Bcl-2 and CD127 than do CD4⁺ naïve and memory T cells [12].

Both RTE and neonatal CD4⁺ cells show a Th2-type cytokine bias in response to in vitro stimulation suggesting that a Th2 profile might be important during the development of the immune system [23-24]. As a precursor of naïve cells we predicted that CD44^{v.low} cells might also be biased towards a Th2 type and away from a pro-inflammatory cytokine profile. We found that CD44^{v.low} cells generate a robust Th2-type response, and are less efficient in generating Th17- and Th22-type responses. In contrast, and consistent with published data, CD44^{int} are very efficient in generating Th17- and Th22-type responses, but not a Th2-type response. CD44^{int} and CD44^{v.low} cells are equivalent in their ability to generate Th1-type responses. Whether the pro-Th2-type cytokine environment generated by CD44^{v.low} cells plays a role in their capacity to inhibit cancer cachexia, a syndrome that is strongly associated with inflammation [25] remains to be determined.

The similarity in phenotype between CD44^{int} T cells and CD44^{v.low} cells suggests that CD44^{v.low} cells are a subset of the naïve cell compartment. In addition to their similarity in expression of CXCR3, IL-2R β , Sca-1, and CD24 they both express a high density of CD45RB and CD62L, and a low density of CD44 [20]. There are also functional similarities between these two populations. Thus, both populations can inhibit lymphopenia by proliferating to replace the lost cells. This process, called LIP, is dependent on CD24 [21]. However, there are several differences in the CD4⁺ T cell pools that are generated by CD44^{v.low} and CD44^{int} T cell subsets indicating that CD44^{v.low} cells are a distinct cell subset. Thus, the CD44^{int} cell-derived T cell pool is significantly smaller than that generated by CD44^{v.low} cells [12]. In addition, CD44^{int} cells are inefficient in reconstituting cells with a naïve phenotype [26] and give rise to a dominantly memory population [27-28] with a pro-inflammatory cytokine profile [17], and limited TCR repertoire diversity [12]. In contrast, CD44^{v.low} cells give rise to a large clonally diverse pool with a balanced ratio of naïve and memory cells, and a non-inflammatory cytokine profile. A functional difference between these two cell subsets is further highlighted by the ability of CD44^{v.low} cells, but not CD44^{int} cells, to inhibit both diabetes and cancer associated cachexia [25].

CD44^{v.low} cells also efficiently generate Foxp3⁺ T regulatory cells whereas naïve cells do not [12]. Tregs can develop either in the thymus (natural or nTregs) [29] or from Foxp3⁻ naïve cells in the periphery (induced or iTregs) [30-31]. A requirement for Tregs in maintaining immune homeostasis is most clearly shown by the multi-organ inflammatory disorder, Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome that ensues if Foxp3 is genetically deficient [29, 32]. Therefore, the capacity to generate functional Tregs is also a critical requirement for optimal precursor cell function.

The level of IFN- γ secreted by CD4⁺ cells from untreated BALB/c mice is significantly higher than for any other cytokine and similar to that secreted by CD4⁺ cells generated by both CD44^{v.low} and CD44^{int} cells. However, compared to untreated BALB/c mice, there is a significant increase in secretion of IL-4 and IL-6, IL-17 and IL-22 by CD4⁺ cells generated by CD44^{v.low} cells and CD44^{int} cells with more IL-4 and IL-6 and less IL-17 and IL-22 secreted by cells from CD44^{v.low} cells. Both IL-4 and IFN- γ have been shown previously to inhibit IL-17 secretion during the development of an immune response [33–34]. It is possible that the lower Th17 response in cells derived by CD44^{v.low} cells compared to CD44^{int} cells is the result of a negative influence by IL-4. IL-6 is a multifunctional cytokine and can play a role in both promoting Th17 differentiation [35–36] and in inhibiting IL-17 secretion from activated T cells [37]. Whether IL-6 plays a different role in T cell development by either CD44^{v.low} or CD44^{int} cells remains to be determined.

5. Conclusions

CD44^{v.low} cells are distinct from RTE and T_{SCM}. Moreover, they generate a CD4⁺ T cell pool that is not only balanced with respect to naïve, memory and Foxp3⁺ Treg cell numbers, but also in its Th2-, Th17-, and Th22-type cell profile. The capacity to secrete Th2 (IL-4), Th17 (IL-17 and IL-22), and Th22 (TNF- α and IL-22) cytokines, and robust secretion of the Th1 cytokine, IFN- γ , is consistent with a role as precursor cells of multiple cell lineages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Berkley AM, Hendricks DW, Simmons KB, Fink PJ. Recent thymic emigrants and mature naïve T cells exhibit differential DNA methylation at key cytokine loci. *J. Immunol.* 2013; 190
2. Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ. Continued maturation of thymic emigrants in the periphery. *Nat. Immunol.* 2004; 5:418–425. [PubMed: 14991052]
3. Haines CJ. Human CD4⁺ T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function. *J. Exp. Med.* 2009; 206
4. Den Braber I, Mugwagwa T, Vrisekoop N, Westera L, Mogling R, et al. Maintenance of peripheral naïve cells is sustained by thymus output in mice but not humans. *Immunity.* 2012; 36
5. Freitas AA, Rocha B. Population biology of lymphocytes: the flight for survival. *Ann. Rev. Immunol.* 2000; 18
6. Jameson SC. Maintaining the norm: T-cell homeostasis. *Nat. Rev. Immunol.* 2002; 2
7. Wu Z, Bensinger SJ, Zhang J, Chen C, Yuan X, et al. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat. Med.* 2004; 10
8. Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Host-reactive CD8⁺ memory stem cells in graft-versus-host disease. *Nat. Med.* 2005; 11

9. Gattinoni, L. L, Zhong XS, Palmer DC, Yi Y, Hinrichs CS, et al. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nat. Med.* 2009; 15:808–813. [PubMed: 19525962]
10. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, et al. A human memory T cell subset with stem cell-like properties. *Nat. Med.* 2011; 17
11. Lugli E, Dominguez MH, Gattinoni L, Chattopadhyay PK, Bolton DL, et al. Superior T memory stem cell persistence supports long-lived T cell memory. *J. Clin. Invest.* 2013; 123:594–599. [PubMed: 23281401]
12. Zhao C, Davies JD. A peripheral CD4+ T cell precursor for naïve, memory, and regulatory T cells. *J. Exp. Med.* 2010; 207
13. Bottomly K, Luqman M, Greenbaum L, Carding S, West J, et al. A monoclonal antibody to murine CD45R distinguishes CD4 T cell populations that produce different cytokines. *Eur. J. Immunol.* 1989; 19
14. Lee WT, Yin XM, Vitetta ES. Functional and ontogenic analysis of murine CD45Rhi and CD45Rlo CD4+ T cells. *J. Immunol.* 1990; 144
15. Swain SL. Generation and in vivo persistence of polarized Th1 and Th2 memory cells. *Immunity.* 1994; 1
16. Firestein GS, Roeder WD, Laxer JA, Townsend KS, Weaver CT, et al. A new murine CD4+ T cell subset with an unrestricted cytokine profile. *J. Immunol.* 1989; 143
17. Cho BK, Rao VP, Ge Q, Eisen HN, Chen J. Homeostasis-stimulated proliferation drives naïve T cells to differentiate directly into memory T cells. *J. Exp. Med.* 2000; 192
18. Lynch HE, Sempowski GD. Molecular measurement of T cell receptor excision circles. *Methods Mol. Biol.* 2013; 979:147–159. [PubMed: 23397394]
19. Budd RC, Cerottini JC, Horvath C, Bron C, Pedrazzini T, et al. Distinction of virgin and memory T lymphocytes: stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J. Immunol.* 1987; 138
20. Zhao C, Wang Z, Robertson MW, Davies JD. Cachexia in the non obese diabetic mouse is associated with CD4+ T cell lymphopenia. *Immunology.* 2008; 125
21. Li O, Zheng P, Liu Y. CD24 expression on T cells is required for optimal T cell proliferation in lymphopenic host. *J. Exp. Med.* 2004; 200
22. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, Polis MA, Haase AT, Feingerg MB, Sullivan JL, Jamieson BD, Zack JA, Picker LJ, Koup RA. Changes in thymic function with age and during the treatment of HIV infection. *Nature.* 1998; 396:690–695. [PubMed: 9872319]
23. Hendricks DW, Fink PJ. Recent thymic emigrants are biased against the T-helper type 1 and towards the T-helper type 2 effector lineage. *Blood.* 117:1239–1249. 201. [PubMed: 21048154]
24. Opiela SJ, Koru-Sengul T, Adkins B. Murine neonatal recent thymic emigrants (RTE) are phenotypically and functionally distinct from adult RTE. *Blood.* 2009; 113
25. Wang A, Zhao C, Davies JD. A novel role for CD4+ T cells in the control of cachexia. *J. Immunol.* 2008; 181
26. Greenberg PD, Riddell SR. Deficient cellular immunity – finding and fixing defects. *Science.* 1999; 285
27. Ernst B, Lee D-S, Chang JM, Sprent J, Surh CH. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity.* 1999; 11
28. Goldrath AW, Bevan MJ. Low affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. *Immunity.* 1999; 11
29. Fontenot J, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 2003; 4
30. Apostolou I, von Boehmer H. In vivo instruction of suppressor commitment in naïve T cells. *J. Exp. Med.* 2004; 199
31. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, et al. Inducing and expanding regulatory T cell populations by foreign antigens. *Nat. Immunol.* 2005; 6

32. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scruffin in CD4+CD25+ T regulatory T cells. *Nat. Immunol.* 2003; 4
33. Park H, Li Z, Yang XO, Chang SH, Nurieva R R, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 2005; 6
34. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, et al. 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
35. Mudter J, Neurath MF. IL-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. *Inflamm. Bowel Dis.* 2007; 13
36. Rose-John S, Mitsuyama K, Matsumoto K, Thaiss WM, Scheller J J. Interleukin-6 trans-signaling and colonic cancer associated with inflammatory bowel disease. *Curr. Pharm. Des.* 2009; 15
37. Zhang S-J, Wang L, Ming L, Guo X-B, Wang H-M, et al. Blockade of IL-6 signal exacerbates acute inflammatory bowel disease via inhibiting IL-17 producing in activated CD4+ Th17 population. *Eur. Rev. Med. Pharmacol. Sci.* 2013; 17

Highlights

- CD4⁺ CD44^{v.low} cells are phenotypically distinct from RTE and T_{SCM}
- CD4⁺ CD44^{v.low} cells are present in spleens of mice six weeks after thymectomy
- CD4⁺ CD44^{v.low} cells from mice that are devoid of RTE show precursor cell activity
- CD4⁺ CD44^{v.low}-derived cells are biased towards generating cells with a Th2-type phenotype
- Foxp3⁺ cells generated by CD44^{v.low} cells have regulatory activity

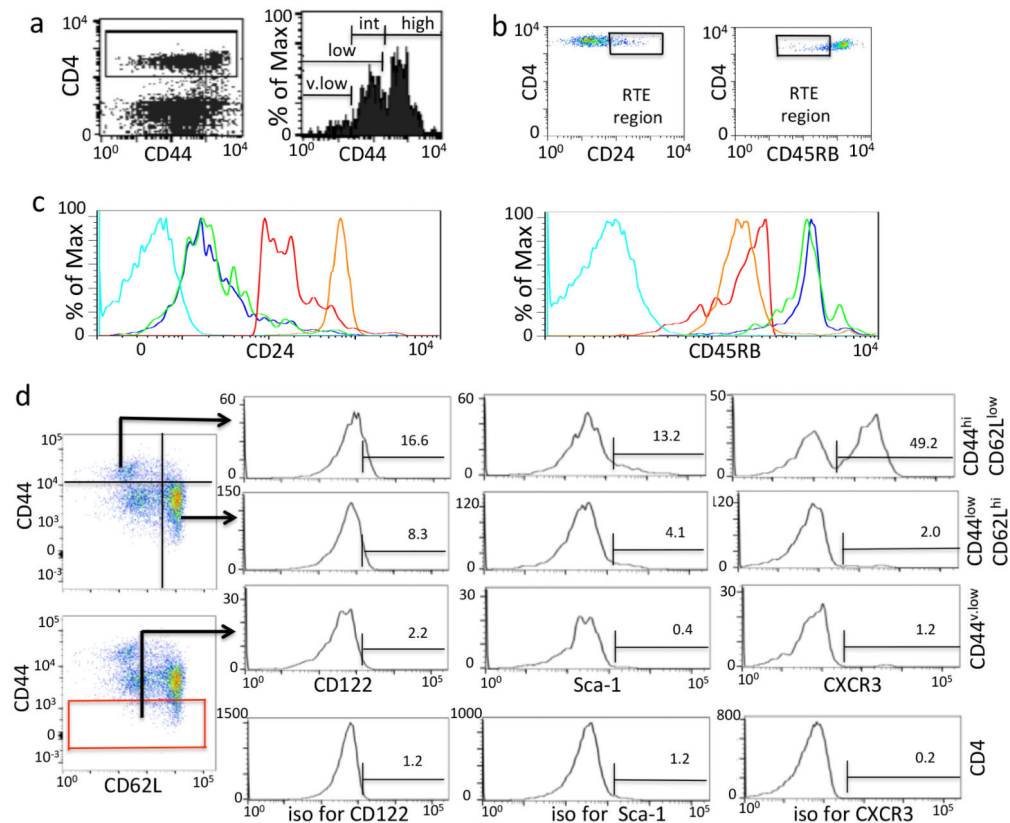


Fig 1. CD4⁺ CD44^{v.low} cells are distinct from RTE and T_{SCM}

Splenocytes and thymocytes from untreated BALB/c mice (n=3–4) were labeled for markers that distinguish RTE and T_{SCM} from mature naïve T cells. (a) Co-expression of CD4 and CD44 on splenocytes. The histogram is gated on CD4⁺ cells and shows the strategy for analysis of CD44^{v.low} cells (v.low), CD44^{int} cells (int), or CD44^{hi} cells (hi). (b) CD44^{low} gated cells identifying the region containing RTE based on expression of CD24^{hi} and CD45RB^{low} (boxes). (c) Expression of CD24 and CD45RB on CD44^{v.low} (green), CD44^{int} (blue), RTE (red), and CD4⁺ single positive thymocytes (orange). Isotype control on CD4⁺ cells is shown in turquoise. The data are representative of all mice analyzed in 3 separate experiments. d) Expression of CXCR3, IL-2R β , and Sca-1 on CD4⁺ CD44^{v.low} cells. Splenocytes were labeled with antibodies specific for CD4, CD44, CD62L, CXCR3, IL-2R β , and Sca-1. Cells were gated on CD4 and the expression of CXCR3, IL-2R β , and Sca-1 on CD44^{hi} CD62L^{low}, CD44^{low} CD62L^{hi}, and CD44^{v.low} is shown in the histograms as indicated. The lowest 3 histograms in the panel show isotype control staining for either CXCR3, or IL-2R β , or Sca-1 on CD4⁺ cells. The data are representative of all mice in two separate experiments.

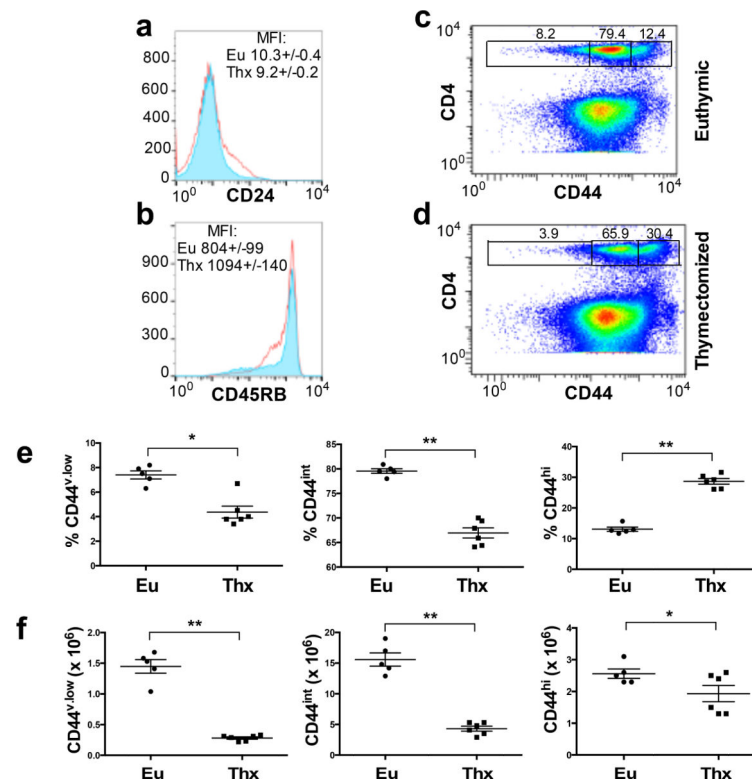


Fig. 2. CD4⁺ CD44^{v,low} cells are present in spleens of mice six weeks after thymectomy BALB/c mice were either thymectomized (n=6) or euthymic (n=5). Six weeks post-thymectomy, spleens were removed and labeled for CD4, CD44, CD24 and CD45RB. The histograms show a representative example of density of expression of CD24 (a) and CD45RB (b) on gated CD4⁺ CD44^{low} (CD44^{v,low} plus CD44^{int}) cells from euthymic (red line) and thymectomized (turquoise) mice. The mean ± SEM fluorescence intensity (MFI) for CD24 and CD45RB expression on CD44^{low} cells from all mice in each group is indicated on each histogram. FACS plots show the co-expression of CD4 and CD44 on splenocytes from euthymic (c) and thymectomized (d) mice, and the results are representative of all mice in each experiment. The rectangles on each plot identify CD4⁺ CD44^{v,low}, CD44^{int}, and CD44^{hi} cells, and the numbers above the rectangles indicate the percentage of total CD4⁺ cells contributed by each subset for that mouse. The relative frequency (e) and total number (f) of CD44^{v,low}, CD44^{int}, and CD44^{hi} cells is shown as the mean ± SEM of one experiment. Each symbol represents an individual mouse. Data are representative of 3 independent experiments. Statistical significance was determined using the Mann-Whitney test. *p<0.05, **p<0.01

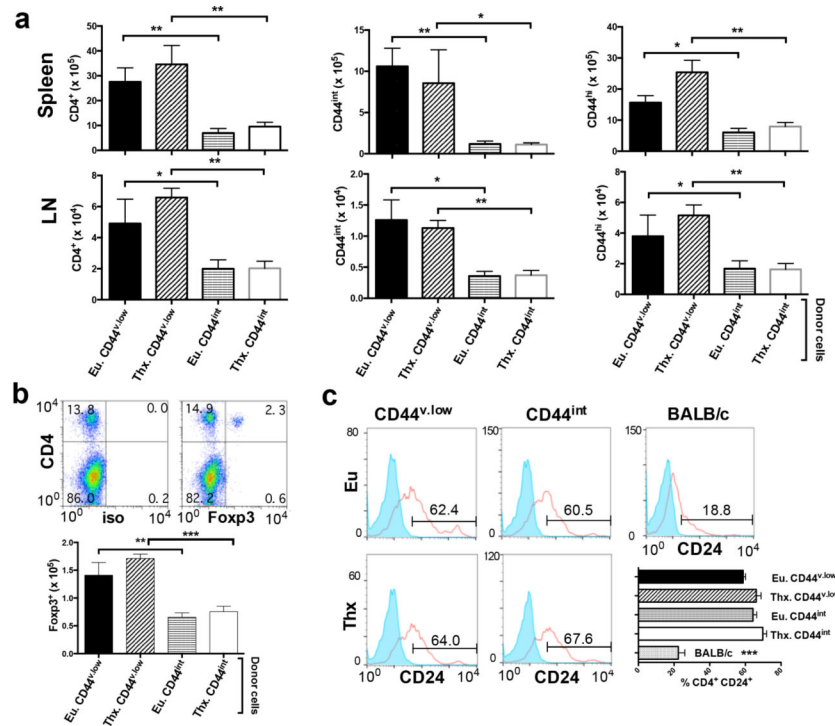


Fig. 3. CD44^{v,low} cells from thymectomized mice show CD44^{v,low} precursor cell activity
 Six-week-old BALB/c mice were thymectomized (n=4) or left untreated (n=4), and 6 weeks later, splenocytes from each group were pooled and co-labeled for CD4 and CD44. CD44^{v,low} and CD44^{int} cells were sorted from each of the two pooled populations and injected iv into CB17.SCID mice to generate 4 groups receiving: CD44^{v,low} from euthymic mice (Eu. CD44^{v,low}, n=7), CD44^{v,low} cells from thymectomized mice (Thx. CD44^{v,low}, n=4), CD44^{int} cells from euthymic mice (Eu. CD44^{int}, n=7), and CD44^{int} cells from thymectomized mice (Thx. CD44^{int}, n=7). Three weeks later, spleens and lymph nodes were isolated, co-labeled for CD4, CD44, and Foxp3 and analyzed by FACS. (a) Total CD4⁺, CD44^{int}, CD44^{hi} cells in spleens and lymph nodes of the indicated mice. (b) Total Foxp3⁺ cells in spleens of the indicated mice. FACS plots show Foxp3 and isotype control staining of splenocytes from a representative Eu. CD44^{v,low} mouse. (c) The relative frequency of CD4⁺ CD24⁺ cells in spleens of the indicated mice compared with untreated BALB/c mice. Histograms are gated on CD4⁺ cells and show CD24 (red lines) and isotype control (turquoise peaks) staining of CD4⁺ cells from either untreated BALB/c mice or from SCID mice injected 3 weeks previously with CD44^{v,low} or CD44^{int} cells from either Eu or Thx donors. Data for all bar graphs (a-c) are the mean ± SEM and are pooled from 2 independent experiments. Statistical significance was determined using the Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001.

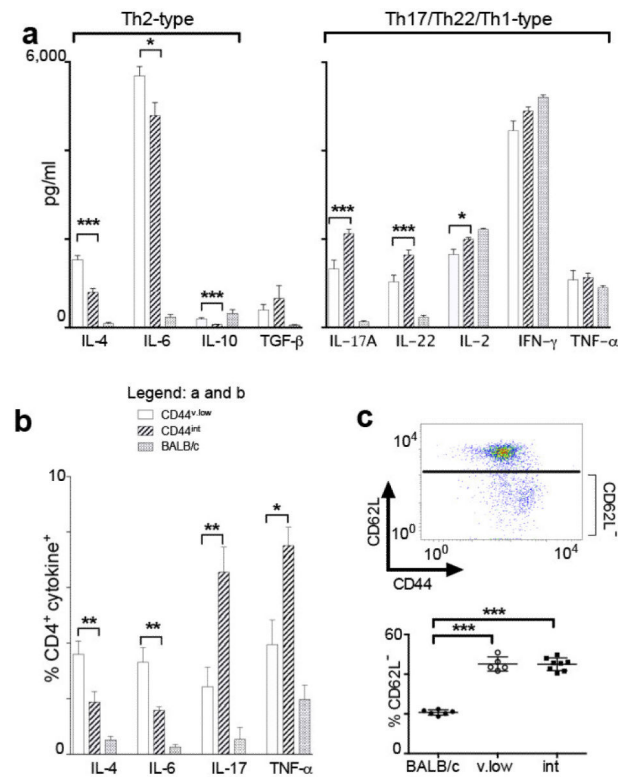


Fig. 4. CD44^{v.low}-derived cells are biased towards generating cells with a Th2-type phenotype
 (a) CB17.SCID mice were injected with sorted CD44^{v.low} cells (n=5) or CD44^{int} cells (n=8). Three weeks later, CD4⁺ cells from each mouse were stimulated in vitro with anti-CD3 and anti-CD28 mAb for 48 h. Th1/Th2/Th17 cytokines in culture supernatants were measured by FACS-based Cytometric Bead Array or, for IL-22 and TGF-β, by ELISA. CD4⁺ cells sorted from an untreated BALB/c mouse were included as controls. Data are the mean ± SEM of triplicates from a single experiment and are representative of 3 separate experiments. Statistical significance was determined using the Mann-Whitney test. (b) CD4⁺ cells enriched from spleens of untreated BALB/c mice (n=6) or SCID mice injected 3 weeks previously with CD44^{v.low} (n=5) or CD44^{int} (n=8) cells were stimulated in culture as described in A. Cells were removed 48 h later and co-labeled for cell-surface CD4 and intracellular IL-4, IL-6, IL-17A, or TNF-α. Data are the mean ± SEM and are pooled from 2 separate experiments. Statistical significance was determined using the Mann-Whitney test, *p<0.05, **p<0.01. (c) As in (b) except cells were stained for cell-surface CD4, CD44, and CD62L. *Upper*: FACS plot showing co-expression of CD62L and CD44 on gated CD4⁺ cells from an untreated BALB/c mouse. *Lower*: Relative frequency of CD4⁺ CD62L⁻ cells in each group. Results are mean ± SEM, and each point represents an individual mouse. Statistical significance was determined using Student's t-test, ***p<0.001.

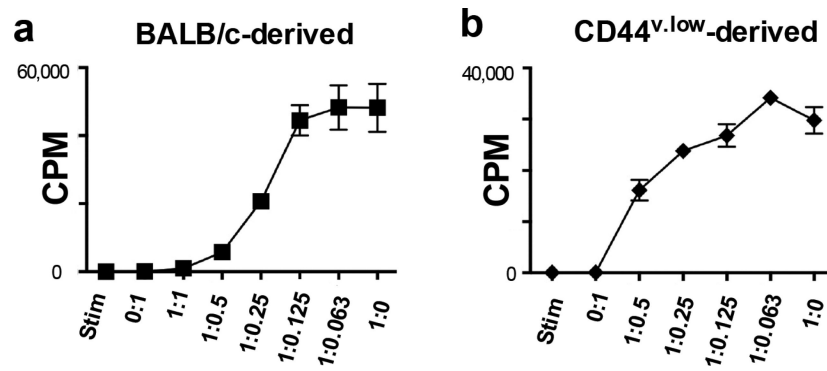


Fig. 5. Foxp3⁺ cells generated by CD44^{v,low} cells have regulatory activity

GFP⁺ Foxp3⁺ cells were sorted from BALB/c Foxp3^{EGFP} spleens (a) or from CB17.SCID mice injected 3 weeks previously with CD4⁺ CD44^{v,low} cells purified from BALB/c Foxp3^{EGFP} mice (b). MLR cultures were set up with the indicated ratios of stimulator to regulator cells with BALB/c spleen-derived CD4⁺ CD25⁻ cells as responders, irradiated C57BL/6 splenocytes as stimulators, and control BALB/c Foxp3^{EGFP} Foxp3⁺ cells (a) or precursor cell-derived Foxp3⁺ cells (b) as the regulators. Proliferation was determined after incubation for 4 days. Data are the mean \pm SD of triplicates.

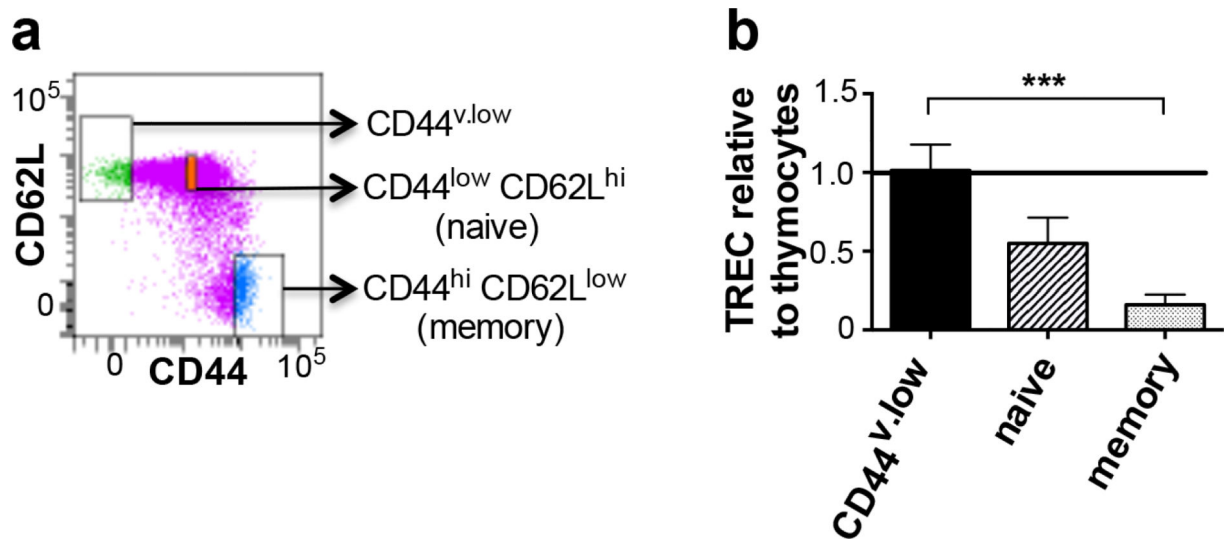


Fig. 6. CD44^{v,low} precursor cells contain the same number of TREC as CD4 single positive thymocytes

CD44^{v,low} precursor cells (CD44^{v,low}, n = 7), CD44^{low} CD62L^{hi} naïve cells (naïve, n = 4), and CD44^{hi} CD62L^{low} memory cells (memory, n = 7) were sorted from CD4 enriched splenocytes of individual untreated BALB/c mice. Single positive CD4⁺ CD3⁺ thymocytes were sorted directly from total thymocyte preparations from the same mice (thymocytes, n = 3). DNA was extracted and TREC content determined for each cell subset from each mouse separately by qPCR. The dot plot shows CD44 and CD62L co-expression on gated CD4⁺ CD3⁺ splenocytes (A). The rectangles show the gates used to sort CD44^{v,low}, naïve and memory cells. Data are presented as mean ± SEM number of TREC relative to single CD4⁺ positive thymocytes (B). Data are pooled from two separate experiments. Statistical significance was determined using the Student t test, ***p=0.0004.