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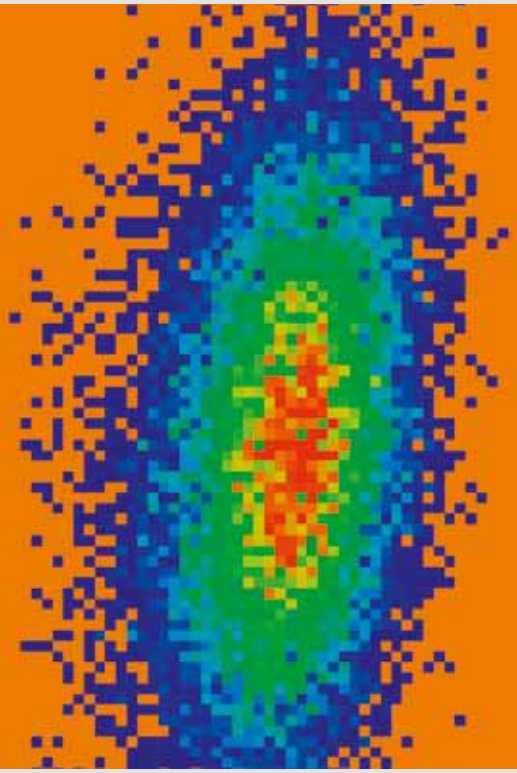

TH cells isolated *ex vivo* maintain memory for interleukin 17 expression

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Introduction

Upon restimulation TH memory lymphocytes re-express distinct cytokines. Originally, two types of TH effector memory cells had been defined: T helper type 1 (TH1) and TH2 cells that re-express interferon γ (IFN-γ), and interleukins (IL)-4, -5, -13, respectively[1]. Recently, a third type of TH effector memory cells has been discovered, which are characterized by the re-expression of IL-17A, IL-17F, and IL-22 (reviewed in ref. 2). This type of cells, termed TH17, can induce autoimmune inflammation[3] and exert a protective function in response to fungal infection[4]. Naive mouse TH cells can be induced to differentiate *in vitro* into TH17 cells by combined TGF-β and IL-6 signaling[5,6], whereas IL-23 promotes survival and proliferation of TH17 cells[6]. In human TH cells, similar signals are required for the differentiation of TH memory cells re-expressing IL-17[7–9]. STAT3 is involved as a signal transducer and IRF-4[10], RORγt[11], and RORα[12] as transcription factors controlling lineage development.

As part of their functional memory, TH1 cells stably re-express IFN-γ and TH2 cells re-express IL-4 and IL-10[13,14]. Cytokine memory for IFN-γ and IL-4 is based on epigenetic modification of their respective genes and expression of the transcription factors T-bet and GATA-3, for TH1 and TH2 differentiation, respectively (reviewed in ref. 15).

To evaluate the cytokine memory of TH17 cells, we have isolated TH cells according to the secretion of IL-17 and analyzed their cytokine memory upon further stimulation *in vitro*. IL-17–secreting cells generated *in vitro* by TGF-β, IL-6, and IL-23 in the absence of IFN-γ and IL-4, up-regulate the lineage-specific transcription factors RORγt and RORα but do not re-express IL-17 in the absence of the original inducing signals. By contrast, IL-17–expressing memory TH cells isolated *ex vivo* re-express IL-17, even when restimulated in the absence of IL-17–inducing signals or in the presence of IL-4 or IL-12.

Materials and methods

Mice

BALB/c and OVA-TCR$^{tg/tg}$ DO11.10 mice (kind gift of Dennis Y. Loh and Kenneth Murphy, Washington University School of Medicine, St. Louis, MO) were bred under specific pathogen-free conditions in our animal facility. The mice were sacrificed by cervical dislocation. All animal experiments were performed in accordance with institutional, state, and federal guidelines.

Antibodies

All antibodies used in these experiments were either conjugated in-house or purchased as indicated. Anti-IL-4 (11B11), anti-IL-12 (C17.18), and anti-IFN-γ (AN17.18.24) antibodies were purified from hybridoma supernatants at the DRFZ and used at 10 mg/mL final concentration. FITC-conjugated anti-CD4 (GK1.5), PE-conjugated anti-IL-17 (TC11-18H10; BD Pharmingen, San Diego, CA) and Cy5-conjugated anti-IL-4 (11B11) were used for all intracellular cytokine stainings.

Cell culture conditions and TH differentiation *in vitro*

CD4$^+$CD62L$^+$ cells from OVA-TCR$^{tg/tg}$ DO11.10 mice (6–8 weeks old) were isolated by using the CD4$^+$CD62L$^+$ T Cell Isolation Kit (Miltenyi Biotec)[13]. All cultures and assays were carried out in RPMI supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.3 mg/mL glutamine and 10 mM β-mercaptoethanol at 37 °C in 5% CO_2. Cell cultures were set up at 3×10^6 cells/mL and stimulated in the presence of 0.5 mM cognate peptide OVA323–339 with irradiated (30 Gy) splenocytes isolated from BALB/c mice as APCs. For TH1 differentiation, cells were stimulated in the presence of recombinant IL-12 (5 ng/mL; R&D Systems, Minneapolis, MN) and anti-IL-4 (11B11) antibody for 6 days. For TH2 differentiation, cells were stimulated in the presence of IL-4 (100 ng/mL, culture supernatant of HEK293T cells transfected with mouse IL-4 cDNA), anti-IL-12 (C17.8) and anti-IFN-γ (AN18.17.24) antibodies. For TH17 differentiation, cells were stimulated in

the presence of TGF-β1 (1 ng/mL), IL-6, IL-23 (20 ng/mL) (all from R&D Systems), anti-IL-4, and anti-IFN-γ antibodies.

Isolation of IL-17–secreting cells *in vitro* and *ex vivo*

Cells were cultured under IL-17–inducing conditions for 6 days, or $CD4^+CD62L^{low}$ cells were isolated from spleen of DO11.10 or BALB/c mice (6 months old). Cells were harvested and restimulated with 10 ng/mL PMA and 1 mg/mL ionomycin (both from Sigma Chemicals) for 1.5 h. The cells were washed twice in ice-cold PBS with 0.5% w/v BSA (PBS/BSA). IL-17–secreting cells were identified by using the Mouse IL-17 Secretion Assay – Detection Kit (Miltenyi Biotec). The $IL\text{-}17^+$ cells and $IL\text{-}17^-$ cells were separated by flow sorting. The purity of sorted cells was confirmed by flow cytometry. Specificity of the IL-17 Secretion Assay was confirmed by intracellular staining following cell sorting.

Intracellular cytokine staining

A total of 4×10^6 cells/mL were stimulated in RPMI medium with 10 ng/mL PMA and 1 mg/mL ionomycin. 5 mg/mL Brefeldin A (Sigma Chemicals) was added after 1 h. After 5 h of stimulation, the cells were washed with PBS and fixed in 2% formaldehyde in PBS for 15 min at room temperature. The cells were stained for intracellular cytokines and analyzed by flow cytometry, as described previously[16].

RNA quantification

RNA preparation and cDNA synthesis was performed as described previously[17]. For details on quantitative real-time PCR see reference 18.

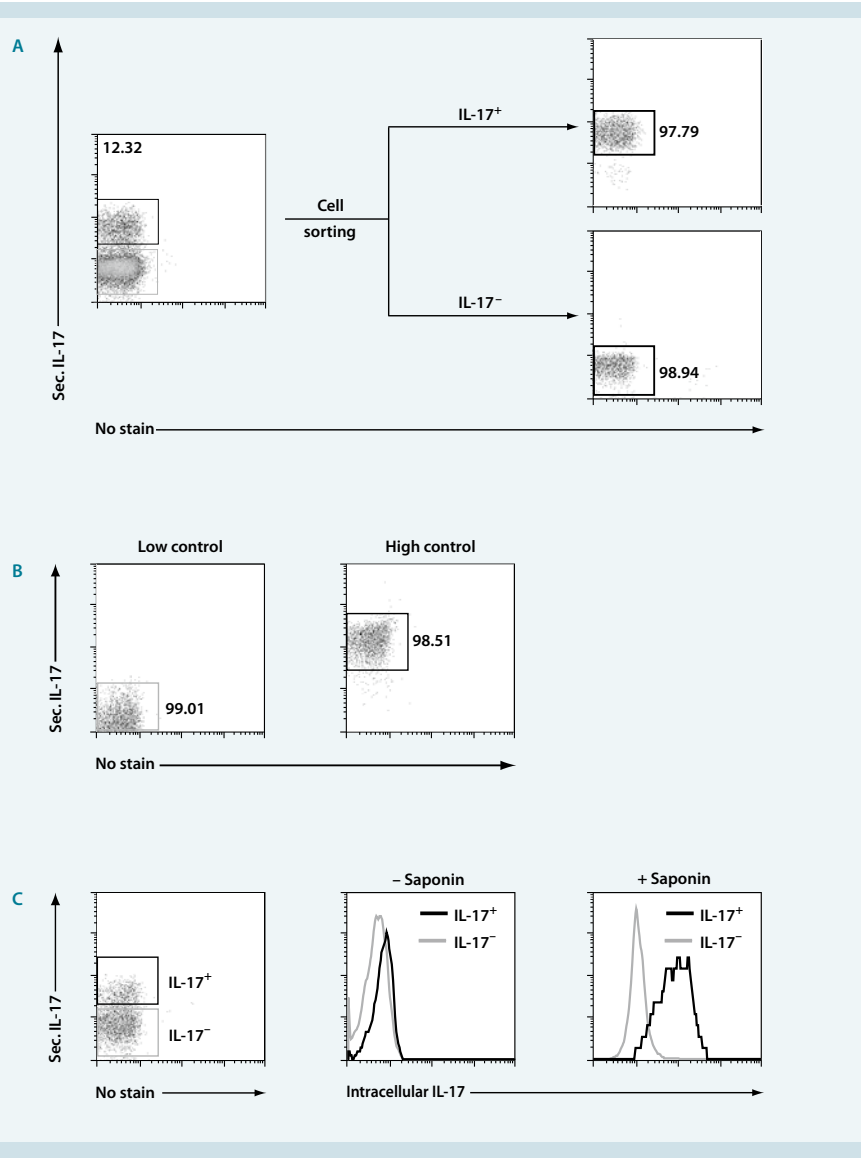

Figure 1 Isolation of viable IL-17–producing and –non-producing cells with the Mouse IL-17 Secretion Assay. (A) Naive $CD4^+CD62L^{high}$ cells were differentiated under IL-17–inducing conditions for 6 days, and the IL-17 Secretion Assay was performed. IL-17–secreting and IL-17–non-secreting cells were separated by flow sorting. (B) After labeling with the IL-17 Catch Reagent, cells were either kept on ice immediately to prevent secretion (low control) or incubated with recombinant mouse IL-17 at RT for 15 min (high control). (C) The staining of secreted IL-17 correlates with the staining of intracellular IL-17. After the IL-17 Secretion Assay, the cells were fixed and stained for intracellular IL-17. To confirm the staining of intracellular IL-17, the staining was performed either in the absence or in the presence of saponin. Data are representative of two experiments.

Results

Direct isolation of IL-17–expressing Th cells

To analyze Th17 memory cells on the single-cell level, we developed a flow cytometric cytokine secretion assay[19,20] for mouse IL-17 (Mouse IL-17 Secretion Assay). Upon restimulation with PMA/ionomycin the maximal frequency of IL-17–expressing Th cells was observed after 1–2 h (data not shown). Accordingly, Th17 cells were restimulated for 1 h to induce cytokine expression, labeled with the IL-17 Catch Reagent, and allowed to secrete IL-17 for 30 min. Subsequently, secreted IL-17 bound to the Catch Reagent was detected by a fluorochrome-conjugated anti-IL-17 antibody. The cells were analyzed by flow cytometry and separated by flow sorting (fig. 1A). As a control, cells were placed on ice for the secretion period, thus blocking secretion (fig. 1B, left plot). The capacity of the Catch Reagent was determined by adding recombinant IL-17 (fig. 1B, right plot). To control for false-positive cells due to binding of IL-17 from secreting cells to the capture matrix of non-secreting cells, the cells from the IL-17 Secretion Assay were fixed and stained for IL-17 in the presence or absence of saponin. All IL-17–secreting but none of the IL-17–non-secreting Th cells were positive for intracellular IL-17 (fig. 1C).

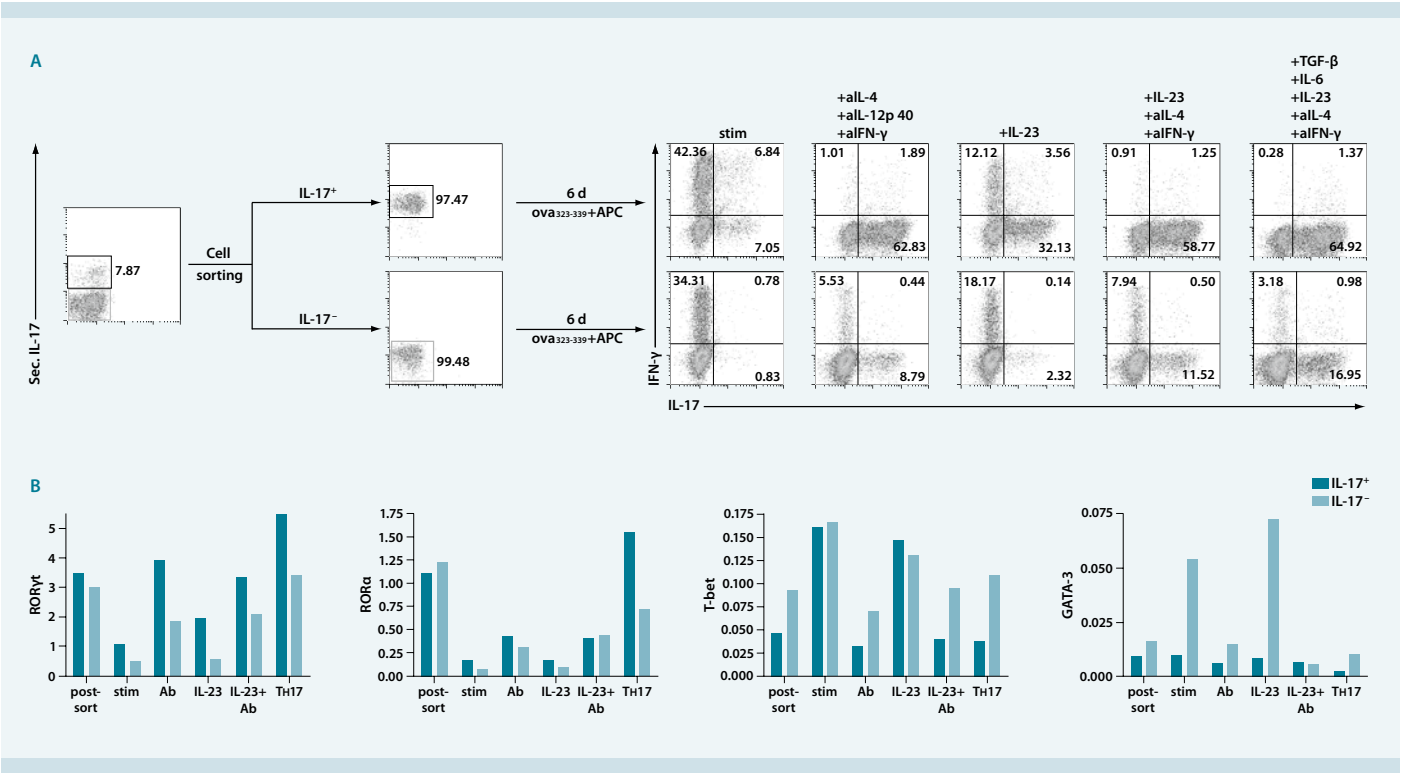

Figure 2 ***In vitro*** **generated TH17 cells fail to re-express IL-17.** Naive $CD4^+CD62L^{high}$ cells from DO11.10 mice were differentiated under IL-17–inducing conditions for 6 days. (A) IL-17–producing and –non-producing cells were separated and recultured for 6 days in the presence of OVA323–339 and irradiated APCs only (stim), under neutral conditions (anti-IL-4, anti-IL-12, anti-IFN-γ) (Ab) or under different TH17–favoring conditions (IL-23, IL-23 with anti-IL-4 and anti-IFN-γ (IL23+Ab) or TGF-β, IL-6, IL-23, anti-IL-4 and anti-IFN-γ (TH17)). Cytokine expression was analyzed by intracellular staining after PMA/ionomycin for 5 h. Data are representative of three experiments. (B) mRNA was extracted from $IL\text{-}17^+$ and $IL\text{-}17^-$ cells directly after isolation (post-sort) and 6 days later from cells restimulated for 2 h, reverse transcribed and quantified by quantitative real-time PCR for RORγt, RORα, T-bet, and GATA-3. Data are representative of two experiments.

IL-17 re-expression is blocked by IL-4 and IFN-γ

We stimulated naive $CD4^+CD62L^+$ cells isolated from DO11.10 mice with antigen in the presence of TGF-β, IL-6, IL-23, anti-IL-4, and anti-IFN-γ to induce TH17 differentiation. After 6 days, the cells expressed IL-17, IL-22, IL-17F, IL-23 receptor, RORγt, and RORα (data not shown). These cells were separated into IL-17–expressing and –non-expressing cells, at purities greater than 97% and 99%, respectively (fig. 2A). In neither cell population could we detect IFN-γ– or IL-4–expressing cells (data not shown). $IL\text{-}17^+$ and $IL\text{-}17^-$ cells were restimulated with the cognate antigen, cultured for another 6 days under various conditions, and analyzed for re-expression of IL-17. Neither of the populations showed selective outgrowth of contaminating cells, when using CFSE to track proliferation of the cells (data not shown). Throughout the culture period cell numbers were comparable and viability was higher than 90%, as evaluated microscopically. When cultured in the absence of exogenous cytokines and blocking antibodies, only 13% of the $IL\text{-}17^+$ TH cells re-expressed IL-17, 49% now expressed IFN-γ (fig. 2A). In the presence of IL-23, 32% of the $IL\text{-}17^+$ cells re-expressed IL-17, and 16% of them expressed IFN-γ. In the presence of blocking antibodies to IL-4 and IFN-γ, more than 60% of the $IL\text{-}17^+$ cells re-expressed IL-17, irrespective of whether IL-23 was blocked by anti-IL-12p40, or was added to the culture. This frequency was also not altered by addition of recombinant TGF-β and IL-6. $IL\text{-}17^-$ cells expressed IFN-γ (35%) but no IL-17 (<1%), if no cytokines or antibodies were added. In the presence of IL-23, 2% of these cells expressed IL-17. Blocking of IFN-γ and IL-4 resulted in the expression of IL-17 in 9% of the cells in the absence of IL-23, 12% in the presence of IL-23, and 18% in the presence of TGF-β, IL-6, and IL-23 (fig. 2A). In $IL\text{-}17^+$ and $IL\text{-}17^-$ cells, RORγt and RORα were expressed at similar levels (fig. 2B) and were down-regulated when the cells were cultured without the addition of antibodies or cytokines. Regulation of RORγt expression corresponded with the expression of IL-17. RORα was generally down-regulated upon reculture, except for a 4-fold up-regulation in the presence of TGF-β/IL-6 compared with cells cultured just in the presence of anti-IL-4, anti-IFN-γ, and IL-23. T-bet expression in $IL\text{-}17^+$ cells was 3-fold enhanced in the presence of endogenous IFN-γ. The expression of T-bet was higher in $IL\text{-}17^-$ than in $IL\text{-}17^+$ cells (fig. 2B).

In vivo generated TH17 cells maintain IL-17 expression *in vitro*

IL-17–expressing TH memory cells generated *in vivo* were isolated directly *ex vivo* from unmanipulated DO11.10 or BALB/c mice (fig. 3). $CD4^+CD62L^{low}$ splenocytes were stimulated with PMA/ionomycin and IL-17–expressing

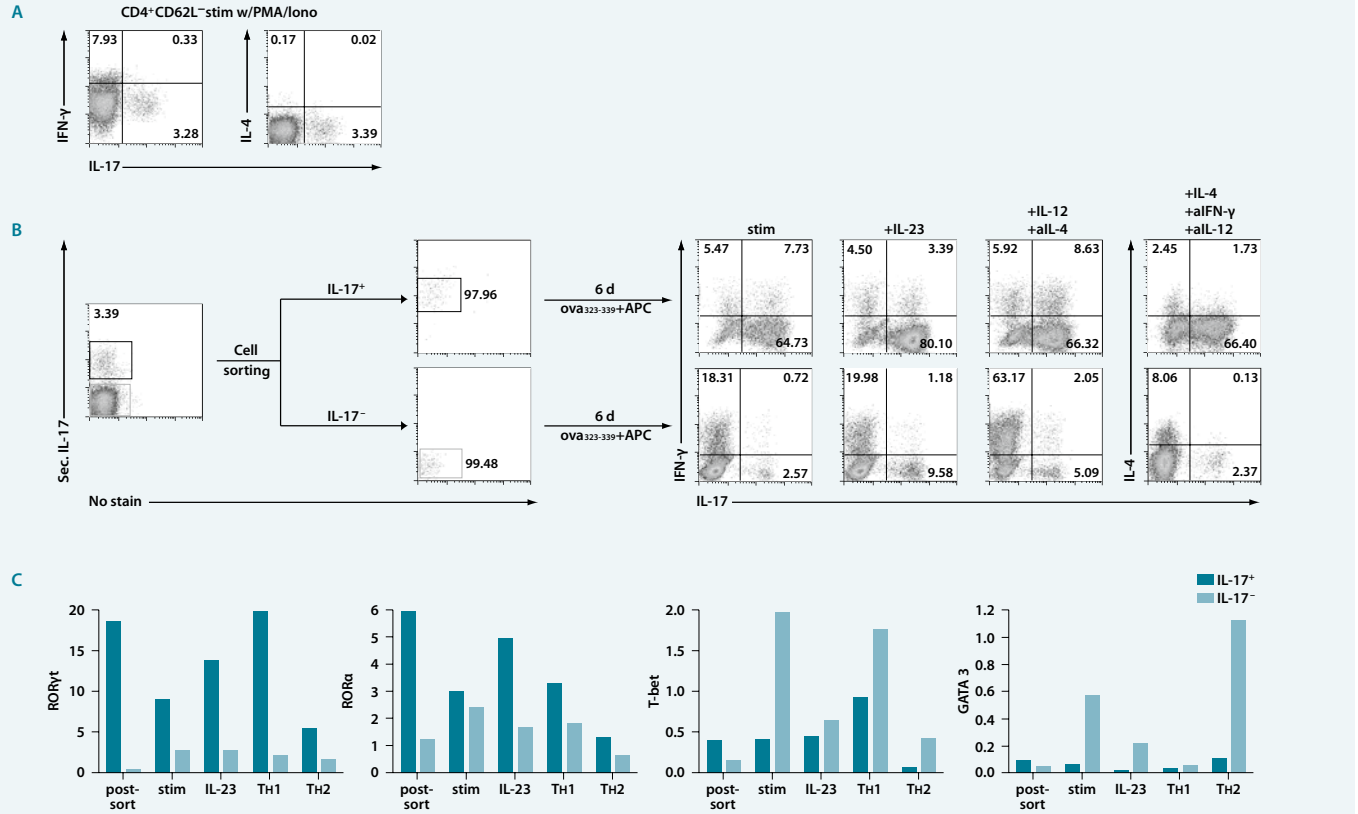

Figure 3 ***In vivo* generated TH17 cells have a stable memory for IL-17.** Memory CD4+CD62L^{low} cells from DO11.10 mice (6 months old) were isolated and either restimulated for (A) direct cytokine analysis or (B) IL-17 Secretion Assay. The IL-17+ and IL-17− cells were cultured without (stim) or with IL-23 and under TH1- or TH2-polarizing conditions for 6 days. The cytokine expression was analyzed by intracellular staining after restimulation with PMA/ionomycin for 5 h. Data are representative of three experiments. (C) mRNA of *ex vivo* isolated IL-17+ and IL-17− cells directly after isolation and cells cultured for 6 days was isolated after 2 h restimulation with PMA/ionomycin, reverse transcribed, and quantified by quantitative real-time PCR. Data are representative of two experiments.

cells isolated with the Mouse IL-17 Secretion Assay. Of the PMA/ionomycin-stimulated cells, 8.2% expressed IFN-γ, 0.2% IL-4, and 3.6% expressed IL-17, of which approximately 25% co-expressed IFN-γ (fig. 3A). Purified IL-17+ and IL-17− cells were recultured for 6 days *in vitro*, either without adding or blocking cytokines, or adding IL-23, or under TH1- or TH2-polarizing conditions (fig. 3B). In the absence of added antibodies or cytokines, 72% of the IL-17+ cells re-expressed IL-17. In the presence of added IL-23, 83% re-expressed IL-17. *Ex vivo* isolated IL-17+ TH cells were refractory to TH1 and TH2-inducing signals: Under TH1 conditions the frequency of IFN-γ–expressing cells was 14%, whereas under TH2-inducing conditions, 4% of IL-4–expressing cells were detected. About 75% and 68% of the IL-17+ cells re-expressed IL-17 under TH1 and TH2 conditions, respectively. The expression of RORγt and RORα was down-regulated 5-fold under TH2 conditions. GATA-3 expression was not induced in IL-17+ cells under any condition. The expression of T-bet was up-regulated 2-fold under TH1-inducing conditions in the IL-17+ cells. In IL-17− cells, RORγt and RORα expression was not up-regulated. T-bet and GATA-3 were induced under neutral and TH1 or TH2 conditions (fig. 3B).

Summary

We have developed a cytometric cytokine secretion assay for mouse IL-17 to analyze the memory of IL-17–expressing TH cells for expression of IL-17.

Our results suggest that the currently available protocols for the *in vitro* induction of IL-17 expression in naive TH lymphocytes lack signals for the induction of a stable memory for IL-17 re-expression. IL-17 expression in naive TH cells was induced very efficiently *in vitro* by TGF-β, IL-6, and IL-23 in the presence of anti-IFN-γ and anti-IL-4 antibodies. However, if IFN-γ or IL-4 were not blocked during subsequent restimulation and culture, no IL-17 re-expression could be observed in cells that once had expressed IL-17. Both IFN-γ and IL-4 are negative regulators of IL-17 expression[21,22]. In *in vitro* generated TH17 cells, the expression of RORγt and RORα was down-regulated under conditions where the cells did not re-express IL-17 (fig. 2B). In summary, IL-17–expressing cells generated *in*

vitro are not functionally imprinted for IL-17 re-expression. Their re-expression of IL-17 depends on the continued presence of the canonical TH17-inducing signals.

By contrast, IL-17–expressing TH cells generated *in vivo* are a stable lineage of effector memory cells. The cells are distinct from TH1 and TH2 cells and functionally imprinted for re-expression of IL-17, even under TH1- or TH2-inducing conditions, i.e., in the presence of IL-12 or IL-4, respectively.

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