Effector memory T helper cells secrete IFN-γ upon stimulation with cytokines: a role in chronic inflammation

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Introduction

Interferon γ (IFN-γ) produced by T helper cells plays a prominent role in both host-protective and pathologic immune responses. Antigen-specific IFN-γ–producing T cells (Th1 cells) are prerequisite for the control and clearance of infections.[1,2] Effector memory Th1 cells are also abundant in chronically inflamed tissues, for example, in rheumatoid arthritis (RA)[3–5]; it is, however, not clear how these cells get activated at the site of chronic inflammation. Recently, it was shown that the proinflammatory cytokines interleukin (IL-)12 and IL-18 synergistically induce IFN-γ production in *in vitro* generated murine Th1 cells in a TCR-independent manner.[6] As the severity of autoimmune diseases such as RA correlates with elevated levels of IL-12 and IL-18[7,8], we hypothesized that the cytokine milieu might be sufficient to induce IFN-γ secretion in Th cells, potentially contributing to inflammation.

We here show that a subset of human resting effector memory Th cells, expressing IL-12R, IL-18Rα, and CCR5 *ex vivo*, secrete IFN-γ upon stimulation via the IL-2R common γ chain in combination with IL-12 and IL-18. CD137 (4-1BB) was identified as a discrimination marker that was only detectable on TCR- but not on cytokine-induced IFN-γ$^+$ Th cells. We were able to detect a significant fraction of Th cells in RA patients' synovial fluid and membrane that spontaneously secreted IFN-γ directly *ex vivo* but lacked CD137 expression, indicating that cytokine-induced IFN-γ$^+$ Th cells operate in chronic autoimmune inflammation.

Material and methods

Cell isolation

CD4$^+$ Th cells were separated from PBMCs using the CD4 MultiSort Kit, human (Miltenyi Biotec). Memory T cells were subsequently isolated by depletion of naive Th cells (CD45RA MicroBeads, human; Miltenyi Biotec) and monocytes (CD14 MicroBeads, human; Miltenyi Biotec). Cell separation was performed using LS Columns or the autoMACS® Separator (Miltenyi Biotec). Purities were higher than 98%.

Viable IFN-γ–secreting cells were detected and isolated using the IFN-γ Secretion Assay – Cell Enrichment and Detection Kit, human (Miltenyi Biotec).

Preparation of single-cell suspensions from synovial fluid and synovial membrane

Mononuclear cells from synovial fluid (SF-MNCs) were washed twice with PBS/BSA containing 2 mM EDTA. Cell debris was removed by using Pre-Separation Filters (Miltenyi Biotec). For the preparation of single-cell suspensions from synovial

membrane (SM) the tissue was minced into pieces of 1 to 5 mm^3, dissociated using the gentleMACS™ Dissociator (Miltenyi Biotec, program spleen _04, followed by brain_03) and digested for 1 h with collagenase IA, hyaluronidase, and DNAse I (Sigma-Aldrich).

Cell culture and stimulation

Cells were cultured in RPMI 1640 with glutamine (Invitrogen) and 10% human AB serum (PAA Laboratories). Recombinant cytokines (R&D Systems) were used at 25 ng/mL unless otherwise indicated. rIL-2 (Roche Diagnostics) was used at 20 U/mL. TCR stimulation was performed by incubation of cells in polysterene tubes coated with anti-CD3 and anti-CD28 antibodies (BD Biosciences) at 0.5 µg/mL and 2.5 µg/mL, respectively.

CMV pp65–specific TH1 cells were generated by culturing PBMCs with 5 µg/mL CMV pp65 Recombinant Protein (Miltenyi Biotec)

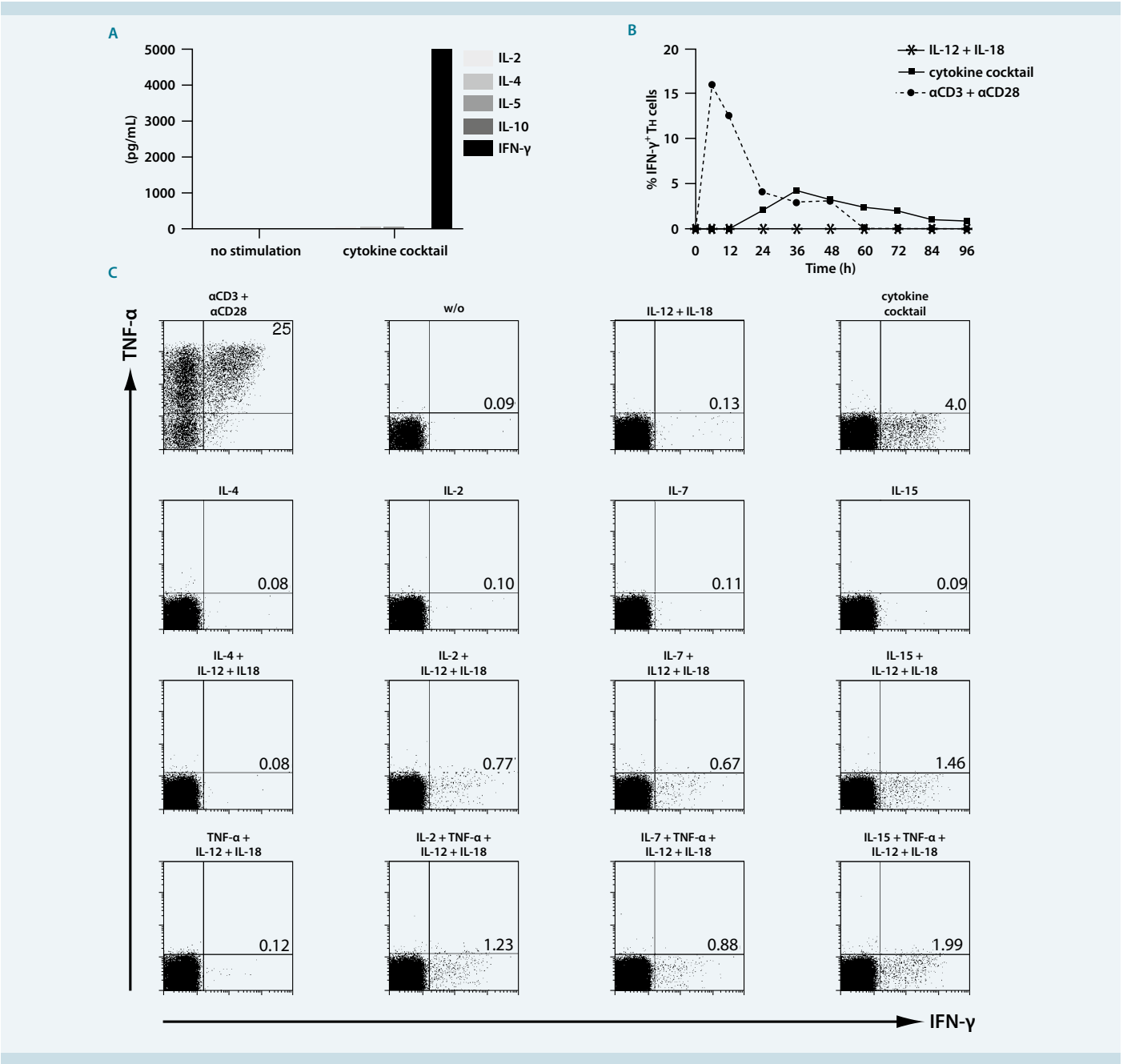

Figure 1 **Induction of IFN-γ secretion in resting human TH cells by inflammatory cytokines.** (A) TH cells were stimulated with the cytokine cocktail containing IL-1β, IL-6, IL-7, IL-8, IL-12, IL-15, IL-17, IL-18, TNF-α, and MIP-1α. After 72 h, supernatants were analyzed for secreted IL-2, IL-4, IL-5, IL-10, and IFN-γ by cytometric bead array (CBA, BD Biosciences). (B) TH cells were stimulated as indicated. Frequencies of IFN-γ–expressing cells were analyzed at different time points intracellularly by flow cytometry. (C) TH cells were stimulated for 36 h with different cytokines or for 12 h with αCD3 + αCD28 and assessed for intracellular IFN-γ and TNF-α production.

and 1 µg/mL anti-CD28 (BD Biosciences) for 6 h. Viable antigen-specific IFN-γ$^+$ cells were stained by using the IFN-γ Secretion Assay (Miltenyi Biotec) and isolated by flow sorting. TH1 cells were expanded for 10 to 14 days in the presence of IL-7 and IL-15 (10 ng/mL each). Antigen-specific restimulation was achieved by culturing 10^5 CMV p65–specific TH1 cells in the presence of 5×10^5 irradiated autologous PBMCs, 5 µg/mL CMV pp65 protein and 1 µg/mL anti-CD28.

Analysis of intracellular cytokines

Stimulated cells were cultured in the presence of brefeldin A for the last 4 to 12 h. Cells were fixed in 2% formalin, permeabilized, stained for 30 min with fluorochrome-conjugated anti-IFN-γ and/or anti-TNF-α and analyzed by flow cytometry.

Results

Resting human TH cells secrete IFN-γ upon stimulation with inflammatory cytokines

To determine whether resting human TH cells are able to secrete IFN-γ in response to cytokines that are present at sites of chronic inflammation, cells were stimulated with a cytokine cocktail comprising IL-1β, IL-6, IL-7, IL-8, IL-12, IL-15, IL-17, IL-18, TNF-α, and MIP-1α for 72 h. Besides IFN-γ, we analyzed secretion of IL-2, IL-4, IL-5, and IL-10. The cells secreted large amounts of IFN-γ; other cytokines were not detectable (fig. 1A). Cytokine-induced IFN-γ production peaked after 36 h of stimulation, whereas TCR-mediated production of IFN-γ showed a maximum between 6 h and 12 h (fig. 1B). After testing all cytokines alone and in combinations to identify the essential components of the cocktail, we found that IFN-γ secretion was induced by the γ-chain cytokines IL-2, IL-7, IL-15, but not IL-4, synergistically with IL-12 and IL-18. IL-15 together with IL-12 and IL-18 proved to be the most effective combination, resulting in frequencies of IFNγ–producing cells of 1.68%+/–0.40% (mean+/–SEM) in TH cells from healthy donors (fig. 1C).

Cytokine induced IFN-γ–secreting cells exhibit a differentiated effector memory phenotype

We isolated human CD45RA$^-$ memory TH cell subsets according to CCR7 and found that the CCR7$^-$ effector memory fraction was highly responsive to cytokine stimulation unlike the CCR7$^+$ central memory subset (data not shown). We then tested whether resting effector memory TH cells express receptors for the cytokines being essential for the IFN-γ response. The IL-18 receptor alpha chain

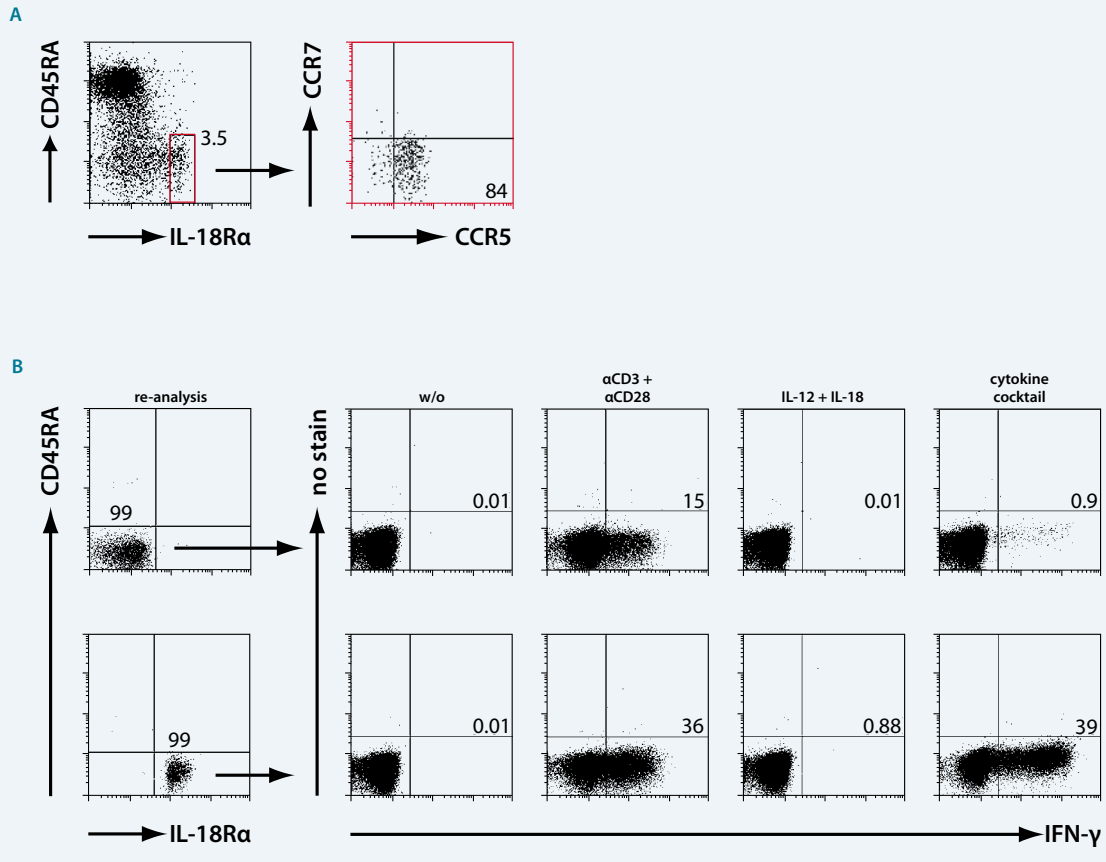

Figure 2 **Cytokine-induced IFN-γ secretion is restricted to CCR7$^-$CCR5$^+$IL-18Rα$^+$ effector memory TH cells.** (A) Assessment of IL-18Rα–expressing memory TH cells among PBMCs and CCR5- and CCR7-expressing cells within the IL-18R$^+$ TH cell population. Gating is indicated in red. One experiment of five is shown. (B) IL-18R$^+$ and IL-18R$^-$ TH cells were analyzed intracellularly for IFN-γ after stimulation for 36 h with the cytokine cocktail or IL-12 + IL-18 or for 12 h with αCD3 + αCD28. One experiment of five is shown.

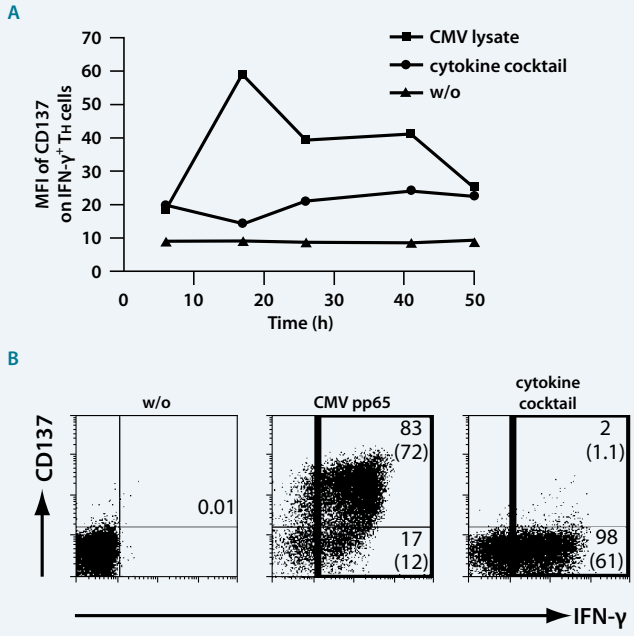

Figure 3 Cytokine-induced IFN-γ⁺ TH cells lack CD137 expression. (A) PBMCs were stimulated with CMV lysate or with the cytokine cocktail. Secreted IFN-γ was detected using the secretion assay on viable IFN-γ⁺ TH cells together with CD137 at the indicated time points. (B) CMV pp65-specific TH1 cells were restimulated with CMV pp65 or with the cytokine cocktail for 14 h. Numbers indicate relative frequencies of CD137⁺ and CD137⁻ cells among the IFN-γ⁺ TH cell population; numbers in parentheses indicate frequencies among total TH1 cells.

(IL-18Rα) was expressed on a small subset of resting TH cells at a frequency of 6.5+/–0.96% (mean+/–SEM). The majority of IL-18Rα^{high} cells was characterized by co-expression of the TH1-associated marker CCR5 and was largely CCR7⁻ (fig. 2A). Up to 40% of the IL-18Rα⁺ cells secreted IFN-γ (33.8+/–2.5%; mean+/–SEM) in response to stimulation with the cytokine cocktail, whereas the IL-18Rα⁻ fraction responded only poorly (0.55+/–0.19%; mean+/–SEM; fig. 2B).

To evaluate the expression of functional IL-12R and IL-15R (being representative for the γ_c signaling cytokine receptors) on sorted IL-18Rα⁺ cells, we analyzed phosphorylation of STAT4 and STAT5, respectively, after 15 min of cytokine stimulation. Detection of both pSTAT4 and pSTAT5 indicated the presence of functional receptors on resting cells *ex vivo* (data not shown).

Strikingly, TH cells from sites of inflammation, i.e., synovial fluid of RA patients share the same phenotypic features, being CD45RA⁻, IL-18Rα⁺, and CCR5⁺ (fig. 4A).

Cytokine-induced IFN-γ⁺ TH cells do not up-regulate CD137 expression

We tested various markers, such as CD69, CD25, CD40L, OX-40, HLA-DR, CD70, and CD137 for expression on TH cells upon stimulation with the cytokine cocktail or via TCR. Only CD137 allowed the distinction between cytokine-stimulated and TCR-induced IFN-γ⁺ TH cells: In CMV lysate–stimulated PBMCs, CD137 expression was detectable after 8 h, peaked at approximately 18 h and lasted up to 48 h in IFN-γ⁺ cells. In contrast, in cytokine-induced IFNγ⁺ TH cells, CD137 remained virtually absent (fig. 3A). We confirmed this observation in a short- term CMV pp65–specific TH1 cell line that was restimulated either by specific antigen or with the cytokine cocktail. Again, CD137 was only induced after specific TCR triggering (fig. 3B). Therefore, CD137 represents a valuable marker to discriminate between cytokine-activated cells and cells stimulated via TCR.

Analysis of IFN-γ⁺ TH cells from RA patients isolated *ex vivo*

The majority of TH cells infiltrating inflamed joints of RA patients are CD45RA⁻CD45RO⁺ IL-18Rα⁺CCR5⁺ effector memory cells as shown in figure 4A. We analyzed these cells for spontaneous IFN-γ production and tested whether cytokine production was induced by inflammatory cytokines rather than (auto-) antigens.

To this end, we used live mononuclear cells from synovial fluid and analyzed them for IFN-γ secretion using the sensitive IFN-γ Secretion Assay technology and for CD137 expression. We found spontaneous IFN-γ secretion in all samples from twelve patients with a frequency of 2.32% +/– 0.39% (mean +/– SEM). This is exemplified in figure 4B (left panel). To also allow an accurate analysis of CD137 expression in samples that contain only few cells spontaneously secreting IFN-γ, we magnetically enriched the cells according to IFN-γ secretion by using the IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (Miltenyi Biotec). Approximately 95% of the enriched IFN-γ⁺ cells did not show CD137 expression (fig. 4B middle panel). As a control, we stimulated SF-MNCs via TCR by a combination of anti-CD3 and anti-CD28 antibodies and found that the cells were capable of expressing CD137.

Moreover, we investigated whether cytokine-induced IFN-γ⁺ cells are also present directly in the inflamed tissue. Cells from synovial membrane were dissociated using the gentleMACS™ Dissociator (Miltenyi Biotec) and analyzed in the same way as SF-MNCs. Cells spontaneously secreting IFN-γ were detectable in all five samples. The majority of these cells showed a cytokine-induced phenotype as they lacked CD137. Similar to the SF-MNCs these cells did not show a general defect in CD137 expression since activation via TCR led to a prominent increase in CD137 expression.

Conclusion

The results presented in this report support the notion that inflammatory cytokines that are abundantly present in chronically inflamed tissues can induce IFN-γ secretion in TH cells. By this mechanism effector memory TH cells can sustain inflammatory processes even without TCR ligation by local (auto-) antigens.

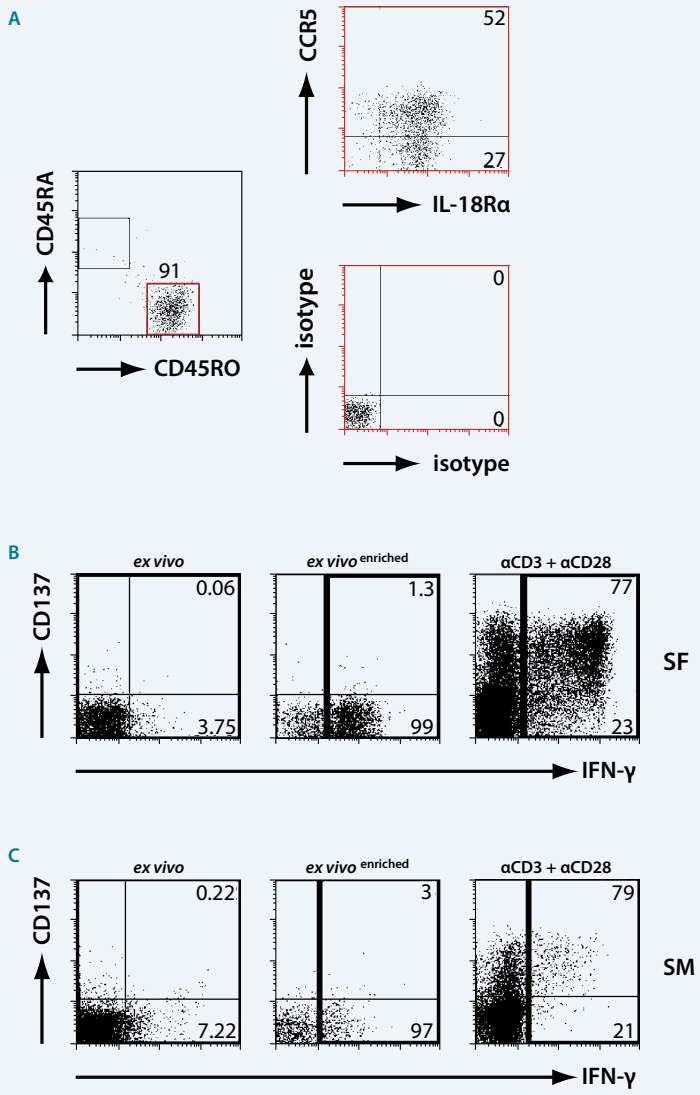

Figure 4 **Synovial IFN-γ⁺ TH cells isolated *ex vivo* from RA patients are induced by cytokines.** (A) Expression of CD45RA, CD45RO, CCR5, and IL-18Rα on SF-derived TH cells *ex vivo*. Gating is indicated in red. (B,C; left panel) viable TH cells from SF (B) or SM (C) were analyzed for IFN-γ secretion using the IFN-γ Secretion Assay and for CD137 expression. Numbers indicate frequencies among TH cells. (B,C; middle panel) viable IFN-γ⁺ cells were magnetically enriched using the IFN-γ Secretion Assay – Cell Enrichment and Detection Kit and analyzed for CD137 expression. Numbers indicate frequencies within the IFN-γ⁺ population. (B,C; right panel) SF-MNCs or SM-MNCs were stimulated for 14 h with a combination of CD3 and CD28 antibodies. CD4⁺ TH cells were analyzed for CD137 and IFN-γ expression. Numbers indicate frequencies within the IFN-γ⁺ population.

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