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A novel, dual cytokine-secretion assay for the purification of human Th22 cells that do not co-produce IL-17A

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Highlights

- The combination of two cytokine-secretion assays allows the sorting of viable IL-22-producing cells as well as viable IL-17A-producing cells.
- For the first time, viable human IL-22-producing Th22 cells, that do not co-produce IL-17A were purified.
- The combination of anti-IL-17A and anti-IL-22 secretion assays is comparable to intracellular staining in the demonstration of Th22 and Th17 cells

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Interleukin-22, Interleukin-17A, human Th22 cells, human Th17 cells, cytokine secretion assay, FACS (fluorescence-activated cell sorting)

Introduction

Adaptive immune responses mediated by CD4⁺ T cells are necessary to eliminate viral, fungal or bacterial infections. They activate other immune cells by releasing T cell cytokines, assist B cells to produce antibodies, and regulate the immune system and tissue cells. CD4⁺ T cells can be characterized according to their cytokine production patterns (1). New subsets of T helper cells were described during the last decade, including Th17 and Th22 cells (2-4). There is still an ongoing debate and no clear distinction between Th17 and Th22 cells in humans, because the main cytokine of Th22 cells, IL-22, can be also produced by Th17 cells. Most of the confusion in the assigning IL-22 as Th17 rather than Th22 cytokine comes from discrepancy between mouse and human data. Among all mouse T helper subsets, Th17 cells are the major source of IL-22 and IL-22 is named as Th17 cytokine (5-7). In contrast, IL-22 production by human cells does not correlate with either ROR γ t or IL-17A (8) and only 10-18% of IL-22-producing T cells in blood co-express IL-17A (2).

IL-22 together with IL-10, IL-19, IL-20, IL-24, IL-26, IL-28A, IL-28B, IL-29 is a member of the IL-10 cytokine family (9). Cells of both, the innate and adaptive arms of the immune system, including $\alpha\beta$ and $\gamma\delta$ T cells, NKT and innate lymphoid cells, can produce IL-22. IL-22 was initially described as a cytokine produced by Th1 cells (10). Although, IL-22 was co-produced by Th17 cells, it was later demonstrated that a fraction of human IL-22-producing T cells do not co-produce IL-17A, IL-4 or IFN- γ , which led to definition of new subset of T helper cells, named Th22 cells (2-4). The main reason why IL-22 is considered a Th17 cytokine is that in mouse models, Th17 cells are the major source of IL-22. To date, characterization of human Th22 cells is mainly performed with *in vitro* manipulated T cell clones or cells expressing a combined chemokine receptor pattern, such as

CCR6⁺CCR4⁺CXCR3⁺CCR10⁺ (2, 4). However, these cells have not been shown to be proven representatives of Th22 cells as CCR10⁺CCR6⁺CCR4⁺ skin homing T cells are only enriched for T cells that produce IL-22 and most of them still co-produce other cytokines.

IL-22 binds to the IL-22 receptor (IL-22R), which consists of two subunits: IL-10R2 and IL-22R1 (11, 12). IL-22 is a unique cytokine that is produced by immune cells and acts solely on non-immune tissue cells (13). In general, the effects of IL-22 on epithelial cells in gut, skin and lungs can be divided into five main categories. Interleukin-22: 1) increases innate defense mechanisms against microbes via the induction of antibacterial protein production by epithelial cells (14-17), 2) influences terminal differentiation of keratinocytes, 3) enhances the production of chemokines by epithelial cells (17), 4) and enhances migratory ability of epithelial cells (15, 18), and 5) shows a self-perpetuating action by upregulation of STAT3 and IL-20 (19, 20).

There is no consensus on the functions of single- and IL-17A/IL-22-co-producing cells so far. Uncontrolled IL-22-production can lead to pathogenesis, as demonstrated in psoriasis (15, 19), atopic dermatitis (21), rheumatoid arthritis (22), and *Toxoplasma gondii* infection (23). One of the factors that may determinate the pro-inflammatory versus protective outcome of IL-22 action is the presence of IL-17A. For example, in the bleomycin induced acute airway inflammation model in mice, where Th17 cells co-produce IL-17A and IL-22, the disease is ameliorated in anti-IL-22 treated WT mice or IL-22^{-/-} mice. Additionally, IL17A^{-/-} knockout mice are protected from airway inflammation, suggesting that IL-17A synergizes with IL-22 in this model (24). These studies explain why it is important to understand the mechanisms of regulation of the production of both cytokines and the effects of single cytokine-producing versus IL-17A⁻ and IL-22-co-producing cells. The present study describes combined IL-17A/IL-22 secretion assays allowing to purify and culture single IL-22-producing-Th22 cells, IL-17A producing-Th17 cells and IL-22, IL-17A-co-producing Th17/Th22 cells. This method may improve our understanding of the physiological and pathogenic functions of cells co-producing cytokines or producing them individually in various diseases, and can be used to identify other cell subsets co-expressing major

cytokines.

Methods

CD4+ T cell isolation

Peripheral blood mononuclear cells (PBMC`s) were isolated from buffy coats and whole blood using density gradient centrifugation (Biochrom, L6155, Berlin, Germany). CD4+ T cells were isolated using an anti-human CD4+ T cells isolation kit (Miltenyi Biotec, 130-096-533, Bergisch Gladbach, Germany) and cultured overnight at 37°C for further experiments in cRPMI 1640 medium (Gibco Cat. No. 21875-034, Paisley, Scotland) supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-Mercaptoethanol (all from Gibco)

Biotinylation of cell surface proteins

Cells surface proteins were biotinylated using Pierce Premium Grade Sulfo-NHS-LC-Biotin (Thermo Scientific, PG82075, Zug, Switzerland) according to the manufacturer`s instructions. Briefly, 10×10^6 cells were washed with PBS pH 8, then stained with 1 mg/ml of Sulfo-NHS-LC-Biotin and incubated for 30 min at room temperature. The dose of Sulfo-NHS-LC-Biotin was optimized in preliminary experiments (data not shown). After incubation, cells were washed 3 times with PBS pH 8 supplemented with 100 mM glycine. Final washing of cells was performed with PBS supplemented with 0.5% bovine serum albumin (BSA) (Sigma, A9418-500G, Buchs, Switzerland) and 2 mM EDTA (Suppl. Fig. 3)

Validation for biotinylation of cell surface proteins

Biotinylated and non-biotinylated cells were stained for 5 min on ice, with phycoerythrin (PE)-labelled streptavidin at a concentration of 6.25 µg/ml (BD Pharmingen, 554061, Allschwil, Switzerland). Both, biotinylated and non-biotinylated cells were then co-stained with 5 µl

(0.25µg) of anti-human CD3 BV421 (Biolegend, 300434, London, UK) antibodies and 5µl (0.25µg) and 7AAD viability dye (BD Pharmingen, 559925, Allschwil, Switzerland) for 10 min on ice. Flow cytometric analysis was performed with BD FACS ARIA II (BD, Allschwil, Switzerland).

Proliferation analysis of biotinylated cells

Equal amounts of PBMCs were biotinylated or left unbiotinylated. All cells were then labelled with cell trace violet dye (CTV; Life Technologies, C34557, Zug, Switzerland). For this, CTV was diluted in pre-warmed PBS (37°C) to a working concentration of 1.25 µM. Cell pellets were resuspended with CTV dye solution at a concentration of 1×10^6 cells/ml and incubated for 20 min at 37°C on a tube rotator to assure uniform labelling. Following this, five times the original staining volume of pre-warmed medium was added and incubated for 5 min to remove non specific CTV dye in the solution. Next, cells were washed and resuspended in cRPMI medium. Biotinylated and non-biotinylated cells were then cultured for 6 days, stimulated with a mixture of anti-CD3, CD2, CD28 mAbs (0.5 µg/ml) or unstimulated. On day 6, cells were stained with Fixable Viability Dye eFluor 780 (eBioscience, 65-0865-14, Vienna, Austria) and anti-human CD3 BV510 antibody (Biolegend, 300448, London, UK).

Streptavidin labelling of anti-cytokine catch antibodies

Purified anti-human IL-22 (eBioscience, 12-7229-42, London, UK) and anti-human IL-17A (eBioscience, 14-7178-85, London, UK) antibodies were labelled with streptavidin using Lightning-Link Streptavidin Conjugation kit (Innova Biosciences, 708-0010, Cambridge, UK) according to the manufacturer's instructions. Total amount of 100 µg of both anti-cytokine antibodies were used for streptavidin labelling.

Titration of anti-cytokine catch antibodies

Streptavidin labelled, anti-cytokine catch antibodies were added to PBMC's at a concentration of 10×10^6 cells in different concentrations, starting from 10 µg/ml, 5 µg/ml, 2.5 µg/ml to 1.25 µg/ml and incubated for 20 min on ice. Next, goat anti-rat IgG (AF488

conjugated, Invitrogen, A11006, Zug, Switzerland) and goat anti-mouse IgG (AF633 conjugated, Invitrogen, 31575, Zug, Switzerland) antibodies were used in 20 µg/ml concentration to detect rat anti-human IL-22 and mouse anti-human IL-17A streptavidin-labelled catch antibodies bound to biotinylated cells surface, respectively. Staining was performed on ice for 20 min. Additionally, cells were stained with anti-CD3 BV421 mAb and 7AAD for 10 min.

Intracellular cytokine staining

After resting overnight, CD3+CD4+ T cells were stimulated in 24 well plates at a concentration of 2×10^6 /ml in cRPMI medium for 4 h with 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, P-8139, Buchs, Switzerland) and 1 µg/ml Ionomycin (Sigma-Aldrich, I-0634, Buchs Switzerland). Following this, 1 µg/ml of brefeldin A (Sigma-Aldrich, B-7651, Buchs, Switzerland) was added for additional 2 h. After stimulation, cells were washed in PBS and stained with fixable viability dye eFluor 780 for 30 min at 4°C (eBioscience, 65-0865-14, Vienna, Austria) followed by staining with 5 µl (0.25 µg) of anti-human CD3 (BV421, Biolegend, 300434, London, UK) and 5 µl (1 µg) of anti-human CD4 for 15 min at 4°C (PE-Cy7, Biolegend, 400325, London, UK). Before intracellular staining, cells were fixed and permeabilized with fixation/permeabilization solution for 20 min at 4°C (BD Cytofix/Cytoperm Fixation/Permeabilization solution kit, 554714, Allschwil, Switzerland). Next, 5 µl (0.25 µg) of anti-human IL-17A AF488 (eBioscience, 53-7179, Vienna, Austria) and 5 µl (0.06 µg) of anti-human IL-22 PE (eBioscience, 12-7229, Vienna, Austria) antibodies diluted in 100 µl of BD Perm/Wash buffer (BD, 554714, Allschwil, Switzerland) were used for intracellular cytokine staining for 30 min at 4°C.

Sorting of IL-17A- and IL-22-secreting CD4⁺ T cells by a dual cytokine secretion assay

After biotinylation as described above, cells were stained with fixable viability dye eFluor 780 for 30 min and anti-human CD3 (BV421, Biolegend, 300434, London, UK) and anti-human CD4 (PE-Cy7, Biolegend, 400325, London, UK) antibodies for 15 min on ice. Next, cells were stained with streptavidinylated anti-IL-22 and anti-IL-17A catch antibodies at a concentration of 10 µg/ml for 20 min on ice. Cells that were not stained with streptavidinylated antibodies served as negative controls. For the cytokine secretion, cells were stimulated with 25 ng/ml PMA, 1 µg/ml Ionomycin in cRPMI medium at the concentration of 1×10^6 and incubated for 4 h on the Tube Rotator (MACSmix Milteneyi Biotec, 130-090-753, Bergisch Gladbach, Germany). After incubation, cells were stained with anti human IL-17A AF488 and anti-human IL-22 detection antibodies for 20 min on ice. It will be good to note here that the same antibodies were used for intracellular staining as well. Four different populations of cells were sorted into PBS based on gates set up with “no-catch antibody reagent” controls:

- 1) IL-17A-producing IL-22-non-producing Th17 cells,
- 2) IL-17A-non-producing and IL-22-producing Th22 cells,
- 3) both IL-17A- and IL-22-producing Th17/Th22 cells and
- 4) cells producing neither IL-22, nor IL-17A.

RT-PCR detection of cytokines in sorted cells

Sorted cells were lysed in RLT Plus RNA lysis buffer (Qiagen, 74034, Hombrechtikon, Switzerland). Total RNA was isolated using RNeasy Plus Micro kit (Qiagen, 74034, Hombrechtikon, Switzerland). mRNA was reverse transcribed with Revert Aid RT Kit (Thermo Scientific, K1691, Zug, Switzerland) according to manufacturer's instructions. Real-time quantitative PCR was performed on a 7900HT Fast Real-Time PCR system (Thermo Scientific, 4329001, Zug, Switzerland) using Maxima Sybr Green/Rox qPCR Master Mix (Thermo Scientific, K0223, Zug, Switzerland) protocol with primers, IL-22 reverse primer: 5'TAG CAG CGC TCA CTC ATA CTG 3', forward: 5'TTC ATG CTC GCCT AAG GAG GC

3', IL-17a reverse primer: 5'CGG ACT GTG ATG TGC AAC CT3', forward: 5' TCC TCA TTG CGG TGG AGA TT3'. Primers sequences used to assess phenotypic differences between

IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} cells are stated in supplementary table 1. Results were normalized to EF1α (eukaryotic translation elongation factor 1 alpha) expression for each sample. Gene expression levels, shown in arbitrary units, were calculated by subtracting EF1 α cycle threshold (Ct) from the gene Ct to get ΔCt value. Arbitrary units for each sample = 10 000 x (2^{-ΔCt}).

Culture of sorted Th22 and Th17 cells

Purified Th22 and Th17 cells were expanded in the presence of 1 nM IL-2 as a growth factor. After 10 days of expansion, T cells were re-stimulated with a combination of anti-CD2, anti-CD3 and anti-CD28 (0.5 ug/ml) monoclonal antibodies in cRPMI medium. Supernatants were harvested after 48 h and IL-22 and IL-17A were measured with human Th17 magnetic bead panel kit (HTH17MAG-14K, Milliplex, Schaffhausen, Switzerland).

Results

Validation of dual cytokine-secretion assay

The complete cytokine secretion assay consists of streptavidin-labelled anti-cytokine catch antibodies, which are bound to the biotinylated surface of the cells, and fluorescently labelled cytokine detection antibodies (Suppl. Fig. 1). Biotin labelling of cell surface proteins is the first step of the novel dual cytokine-secretion assay. To prove that this step was done correctly, the detection of biotinylated cell surface using a PE-labelled streptavidin was performed. As demonstrated in Fig. 1A, only biotinylated cells were stained with streptavidin-PE, as compared to non-biotinylated cells, with approximately 98 % of viable, CD3+ T cells stained with streptavidin-PE, demonstrating that biotinylation of cell surface proteins was successful (Fig. 1A,B). The reagent (Sulfo-NHS-LC-Biotin) that was used to biotinylate the cells is membrane impermeable, water-soluble and biotinylates only primary amines present

on the surface of the cells. In accordance with that fact, 93.14 ± 1.52 % of biotinylated CD3+ T cells were viable, as compared to 96.17 ± 1.52 % of viable non-biotinylated cells, demonstrating that biotinylation had no effect on cell viability (Fig. 1C).

Next, to investigate, if biotinylation activates the cells and induces their proliferation, peripheral blood mononuclear cells (PBMC's) were stimulated with an anti-CD2/CD3/CD28 mixture of mAbs and stained with a cell division tracking (CTV) reagent. On day 6, 94.0 ± 6.6 % of biotinylated and 92.7 ± 6.3 % of non-biotinylated CD3+ T cells proliferated in response to T cell specific stimuli. Furthermore, spontaneous proliferation of non-stimulated, biotinylated cells was not observed (Fig. 1D), (Suppl. Fig. 2). These data prove that surface biotinylation of T cells influence neither their viability nor their ability to proliferate.

After biotinylation, cells were ready for the next step in the cytokine secretion assay procedure: Staining with anti-cytokine catch antibodies. Here, we took the advantage of the fact that streptavidin has a very high affinity for biotin ($K_d = 10^{-14}$) (25), and we labelled purified anti-cytokine catch antibodies with streptavidin and used them to bind to biotin on the surface of the cells. To find the optimal concentration of streptavidin-labelled catch antibodies, which cover the whole cell surface, a 10, 5, 2.5, to 1.25 $\mu\text{g/ml}$ titration of both antibodies was performed (Fig. 2A). To detect these catch antibodies (Ab's) on the surface of the cells, we took the advantage that anti-human IL-22 antibody (Ab) was of rat origin and anti-human IL-17A Ab was of mouse origin. Therefore, we used goat anti-rat IgG AF488 Ab to detect anti-IL-22 catch antibody, and goat anti-mouse AF633 Ab to detect anti-IL-17A catch antibody on the surface of cells (Suppl. Fig. 3). As shown in Fig. 2B, concentration-dependent labelling of the cells was observed, with 1.25 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$ of antibodies not being sufficient to cover the whole surface of cells. Only Ab's at concentration of 5 and 10 $\mu\text{g/ml}$ enabled a complete staining of the cell population. As shown on Fig. 2B, the percentage of cells labelled with both catch antibodies in case of 10 $\mu\text{g/ml}$ concentration reached $97,8 \pm 0.75\%$, we decided to use for further assays anti-cytokine catch antibodies at

a concentration of 10 µg/ml. Dual secretion assay was capable of catching IL-22 and IL-17A cytokines produced by the cells. Secreted cytokines were bound to catch antibodies on the surface of cells and later detected with fluorescently labelled detection antibodies.

IL-22- and IL-17A-secretion assays recognize cytokine-producing CD3+CD4+ T cells in similar quantities compared to intracellular cytokine staining

To analyse intracellular cytokine production, cells were gated on viable CD3+CD4+ T cells and IL-22 and IL-17A production was determined (Fig. 3A). As presented in Fig. 3B, the percentage of IL-22^{pos} cells detected with secretion assay positively correlated with the percentage of IL-22^{pos} cells identified with intracellular staining. More interestingly, similar correlation, was found for Th22 cells, which produced IL-22 and didn't produce IL-17A. In addition, positive correlation between secretion assay and intracellular staining was observed for IL-17A-producing cells: IL-17A^{pos}IL-22^{pos} cells and IL-17A^{pos}IL-22^{neg} cells. We were able to detect cell populations as low as 0.05% and 0.06% of IL-22+ and IL-17A+ T helper cells with the dual secretion assay (Suppl. Fig. 4), suggesting that this secretion assay is as sensitive as intracellular cytokine staining.

Purified Th22 cells are characterized by high expression of IL22, but not IL17a mRNA

In the next step, IL-17A-single positive, double IL-17A/IL-22-producing, IL-17A/IL-22 double negative and most importantly, IL-22-single positive cells were sorted (Suppl. Fig. 4A). During sorting, cells that were not stained with anti-cytokine catch antibodies, but were stained with fluorescently labelled detection Ab's, served as controls. Based on these controls, gates were set for better estimation of cytokine production by T cells. To avoid cytokine-non-producing cells non-specifically binding the cytokines produced by neighbouring cells, the cells were suspended at a concentration of 1x10⁶/ml and incubated on a tube rotator to provide constant mixing of cells, thereby avoiding cell-cell contact. Although, the production of both cytokines varied between different donors, staining with

secretion assays allowed sorting of 4 separate populations of cells. The percentage of IL-22^{pos}IL-17A^{neg} T cells was significantly higher than the percentage of IL-22^{pos}IL-17A^{pos} T cells ($p \leq 0.001$) suggesting that there are more Th22 cells than Th17/Th22 cells in peripheral blood (Fig. 4A).

Subsequently, mRNA was isolated from sorted cells and the expression of IL22 and IL17A mRNA was quantified by RT-qPCR (Fig. 4B). IL-22 expression was only detected in IL-22 single positive and IL-17A/IL-22-double positive cells, but not in IL-17A-single positive and IL-17A/IL-22-double negative cells. Similar results were obtained for IL-17A mRNA expression, with IL-17A detected only in IL-17A-single positive and IL-17A/IL-22-double positive cells. Interestingly, IL22 mRNA was significantly higher expressed in IL-22-single positive and double positive cells as compared to double negative cells. Moreover, analogous to this finding it was reported for IL17A mRNA expression, as IL17A was expressed significantly higher in IL-17A-single positive as well as in double positive cells when compared to double negative cells.

In addition, the expression of chemokine receptors, T helper cell-related transcription factors and cytokines was analyzed in IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} sorted T cells (Supplementary Figure 6). IL-22^{neg} IL-17^{pos} cells expressed higher mRNA levels of CCR6 and CCR4 chemokines receptors and RORC2 transcription factor compared to IL-22^{pos} IL-17^{neg} cells. There was no difference in mRNA expression of CCR10, CXCR3, CLA, T-bet, Gata-3, AHR, IFN- γ and IL-4 between IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} T cells

Furthermore, IL-22^{pos}IL-17A^{neg} Th22 cells were purified and expanded in culture for 12 days. They were characterized by production of IL-22 and little or no production of IL-17A (Fig. 4C). A similar finding was observed for IL-22^{neg}IL-17A^{pos} Th17 cells, which did not produce IL-22, but produced IL-17A (Fig. 4C). In conclusion, these results confirm that dual secretion assay detects accurately Th22 as well as Th17 cells, and sorted Th22 and Th17 cells show a stable phenotype over the course of 12 days in culture

Discussion

The first cytokine secretion assay was established in 1995 by Manz and colleagues to sort viable cells according to their secreted molecules, secreted to a cell-surface affinity matrix (26). Since then, some commercially available secretion assays were developed and consist of anti-cytokine catch reagent (anti-CD45 Ab linked to an anti-cytokine Ab) and detection Ab binding to a different epitope of the cytokine of interest, which is labelled with a fluorescent dye. The main restriction of above-mentioned secretion assays is that limited number of fluorescently labelled detection antibodies exists and IL-22-secretion assay has not been developed yet.

For a long time, IL-22-producing cells were considered as a subset of Th17 cells (7). This was mainly due to the fact that, there are only a few tools available to study human Th22 cells, such as intracellular cytokine staining, generation of T cell clones and cell sorting based on chemokine receptors (2-4). In the present study, we show that a combination of dual anti-IL-22 and anti-IL-17A secretion assays, allow to sort viable IL-22-producing Th22 cells, that do not co-express IL-17A. This novel method expands the possibilities to further characterize human Th22 cells, because cells sorted with this secretion assay can be sterile handled and characterized, co-cultured with other cells or used in humanized mouse models.

One of the novel and important steps in this currently presented dual-secretion assay is the biotinylation of cell surface proteins. Our data show that biotinylation of cells did not affect cell viability and the ability of cells to proliferate without any reservation. Moreover, biotinylated cells do not proliferate spontaneously. Together, these results suggest that, sorted, biotinylated cells can be cultured *in vitro*.

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In the next step, streptavidin labelled anti-cytokine antibodies were used to catch cytokines produced by cells. At this step, it was possible to combine more than two anti-cytokine catch antibodies, as long as detection antibodies used in next step recognize different epitopes of the same cytokine and were labelled with different fluorochromes. In the present study, two catch antibodies were used, which allowed us to sort Th22 cell that do not express IL-17A and Th17 cells that do not express IL-22. After culture, the sorted cells kept their profile of the initial sorting strategy with Th22 cells producing IL-22 and low amounts of IL-17A, and Th17 cells producing IL-17A and not producing IL-22.

The measurement of transcription factors and chemokines provided further evidence on the purity and functional relevance of Th17 and Th22 cells. IL-22^{neg} IL-17^{pos} T cells expressed higher levels of CCR6 and CCR4 chemokine receptors compared to IL-22^{pos} IL-17^{neg} cells suggesting that previously reported pattern of chemokines receptors expression - CCR6⁺CCR4⁺CXCR3⁻ (2, 4), better describes Th17 rather than Th22 cells. As expected, IL-22^{neg} IL-17^{pos} expressed higher levels of RORC2 transcription factor compared to IL-22^{pos} IL-17^{neg} T cells. AhR, has been reported to be essential for IL-22 expression in Th17 cells (27, 28), $\gamma\delta$ T cells (29) and human Th22 cells (2, 4) in some studies, whereas others have reported that AHR is not absolutely required for IL-22 expression (30). Although it was expressed in relatively high levels in both cell subsets, AhR expression was not different between IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} cells in the present study.

To further validate the cytokine profile and quality of purified cells, IL-17A and IL-22 production by T cells was compared between intracellular staining and the secretion assay. During intracellular staining, permeabilization and fixation of cells renders them useless for further functional experiments. In addition, protein transport from endoplasmic reticulum (ER) to Golgi apparatus is blocked with brefeldin A during intracellular staining. This method gives only information about the ability of cells to produce cytokines, without revealing their extracellular secretion. In contrast, cell sorting based on their cytokine production with a

secretion assay gives a capability to culture viable cells and study their biology in detail. The cells detected with a secretion assay are the cells, which secrete cytokines into the extracellular space resembling more closely the *in vivo* situation. For both presented methods, cells were stimulated with PMA and ionomycin, but in case of the secretion assay stimulation took place after surface staining, during incubation on tube rotator at 37°C, and in case of intracellular staining, it took place before surface staining and was followed by the addition of brefeldin A. Nevertheless, the percentages of cells detected with the secretion assay significantly correlated with percentages of cells detected with intracellular staining, suggesting that secretion assay is as sensitive in the detection of cytokine-producing cells as intracellular cytokine staining.

In conclusion, for the first time, we were able to sort pure, human Th22 cells based on their IL-22 production which are lacking IL-17A production. Sorted cells could be cultured at a later stage and their cytokine profile investigated. The combination of two secretion assays,

as presented here, gives a possibility to sort different subsets of Th22 cells, co-producing other cytokines, such as IL-17A and investigate the interaction of these cells with other cells. Understanding the differences in the regulation of IL-22 may provide an insight into the pathogenic and physiological functions of this cytokine in various diseases. This methodology will enable even more than two cytokines to be analysed at the same time. As soon as the pair of antibodies, which detect different epitopes of the same cytokine exist, and one of them is labelled with a fluorescent dye, multiple anti-cytokine secretion assays may be combined for more precise analyses of single or combined cytokine-secreting cells.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Figure 1. Validation of cell surface biotinylation.

Biotinylated and non-biotinylated PBMCs were stained with streptavidin-PE to examine the efficiency of biotinylation process (A). In the gate of viable CD3+ T cells, $97.32 \pm 4.52\%$ of biotinylated CD3+T cells were stained with streptavidin - PE (B). The biotinylation of cells does not influence cells viability, as the percentages of viable CD3+T cells were similar among biotinylated and non-biotinylated cells (n=3) (C). Next, proliferation of biotinylated and non-biotinylated cells in response to anti-CD2, CD3, CD28 mAb stimulation was investigated. Similar percentages of biotinylated and non-biotinylated CD3+T cells responded to anti-CD2/CD3/CD28 stimulation on day 6 after starting the cultures as measured using the cell proliferation dye (CTV). Biotinylation of the cell surface did not induce spontaneous proliferation of biotinylated cells (n=4)(D). Two-way Anova test with multiple comparisons was applied ($p < 0.0001$).

Figure 2. Titration of anti-cytokine catch antibodies binding to the cell surface.

Binding of streptavidin-labelled anti-cytokine catch antibodies to biotinylated cell surface was analysed, and the titration of the anti-cytokine catch antibodies was performed (A). Rat anti-human IL-22 and mouse anti-human IL-17A catch antibodies were detected with goat anti-rat AF488 and goat anti-mouse AF633 antibodies, respectively (B). Concentration-dependent labelling was observed, and optimal dose of 10 $\mu\text{g/ml}$ of both catch antibodies was established (n=4). One-way Anova test with multiple comparisons was calculated.

Figure 3. Comparison of cytokine production detected by secretion assays and intracellular staining.

The production of IL-17A and IL-22 was analysed using both, the secretion assay and intracellular while gating on staining viable, CD3+CD4+T cells (A). The percentage of CD4+IL-22+, CD4+IL-17A+ T cells detected in secretion assay correlated with results obtained with intracellular staining (B). A strong correlation was observed for IL-17A^{neg}IL-22^{pos} Th22 cells, IL-17A^{pos}IL-22^{pos} Th17/Th22 cells and for IL-17A^{pos}IL-22^{neg} Th17 cells (B). Each dot represents a separate donor (n=7). Non parametric Spearman correlation coefficient r was calculated.

Figure 4. Sorting and characterization of IL-22-producing Th22 cells and IL-17A-producing Th17 cells.

CD3+CD4+T cells were stained with anti-IL-17A and IL-22 secretion assays and four different populations of cells were sorted (IL-22^{pos}IL-17A^{neg}, double positive, IL-17A^{pos}IL-22^{neg}, double negative), expression of IL17A and IL22 was measured with RT-PCR (B). IL22 expression was only detected in IL-22^{pos}IL-17A^{neg} and double positive cells. Whereas, expression of IL-17A was detected only in double positive and IL-22^{neg}IL-17A^{pos} T cells (n=5) (B). The percentage of IL-22 positive cells, which do not produce IL-17A varied from 0.074% to 0.72% of total CD3+CD4+ T cells (A) (n=7) and were significantly higher in number as compared to IL-17A/IL-22 double positive cells. No difference in number of CD4+IL-17A+ and CD4+IL-22+ cells was observed. Sorted IL-22^{pos}IL-17A^{neg} and IL-22^{neg}IL-17A^{pos} cells were cultured in cRPMI medium supplemented with IL-2 for 12 days and after the second round of T cells re-stimulation with anti-CD2/CD3/CD28 antibodies, production of IL-22 and IL-17A was measured by luminometric bead array. Th22 cells produced IL-22 with a very low amount of IL-17A, in contrast Th17 cells produced IL-17A with no-co production of IL-22 (C). One-way Anova test for repeated measures was applied.

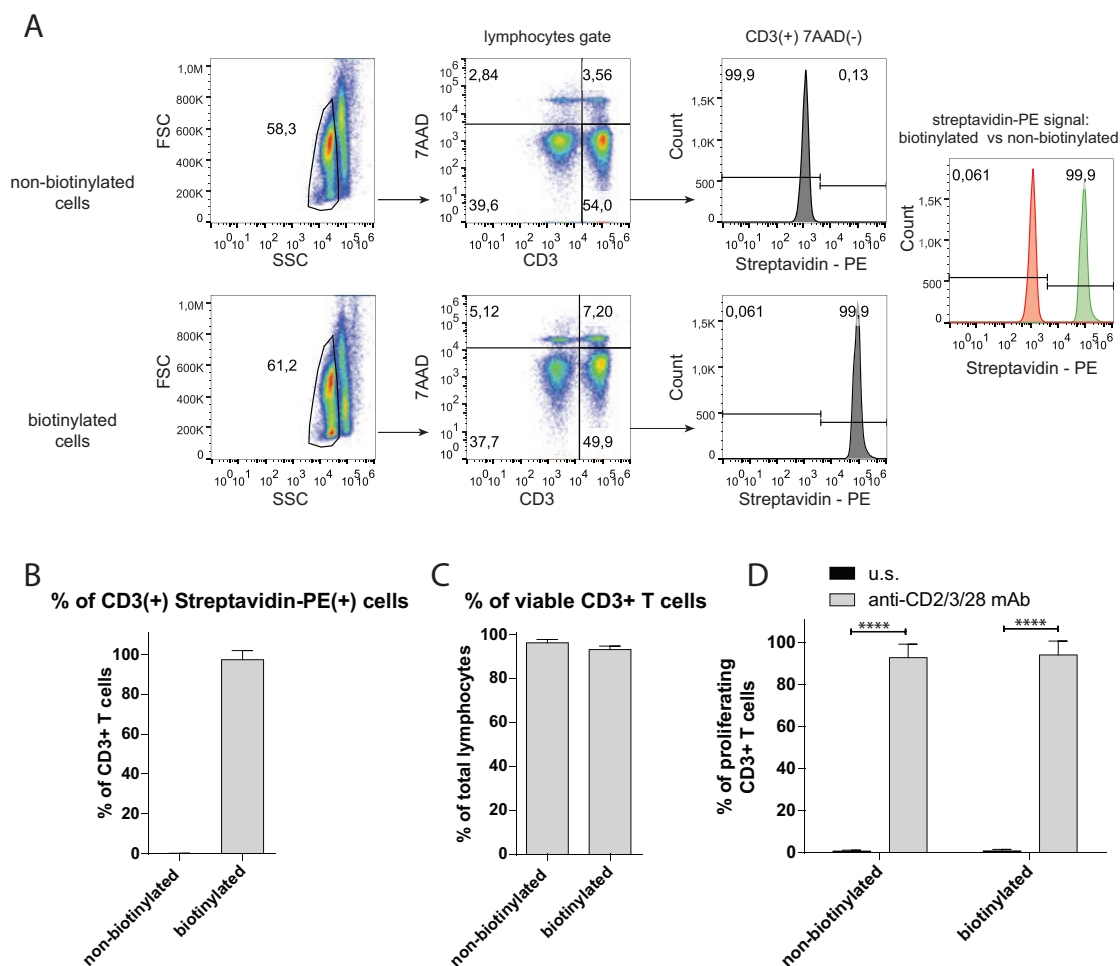


Figure 1.

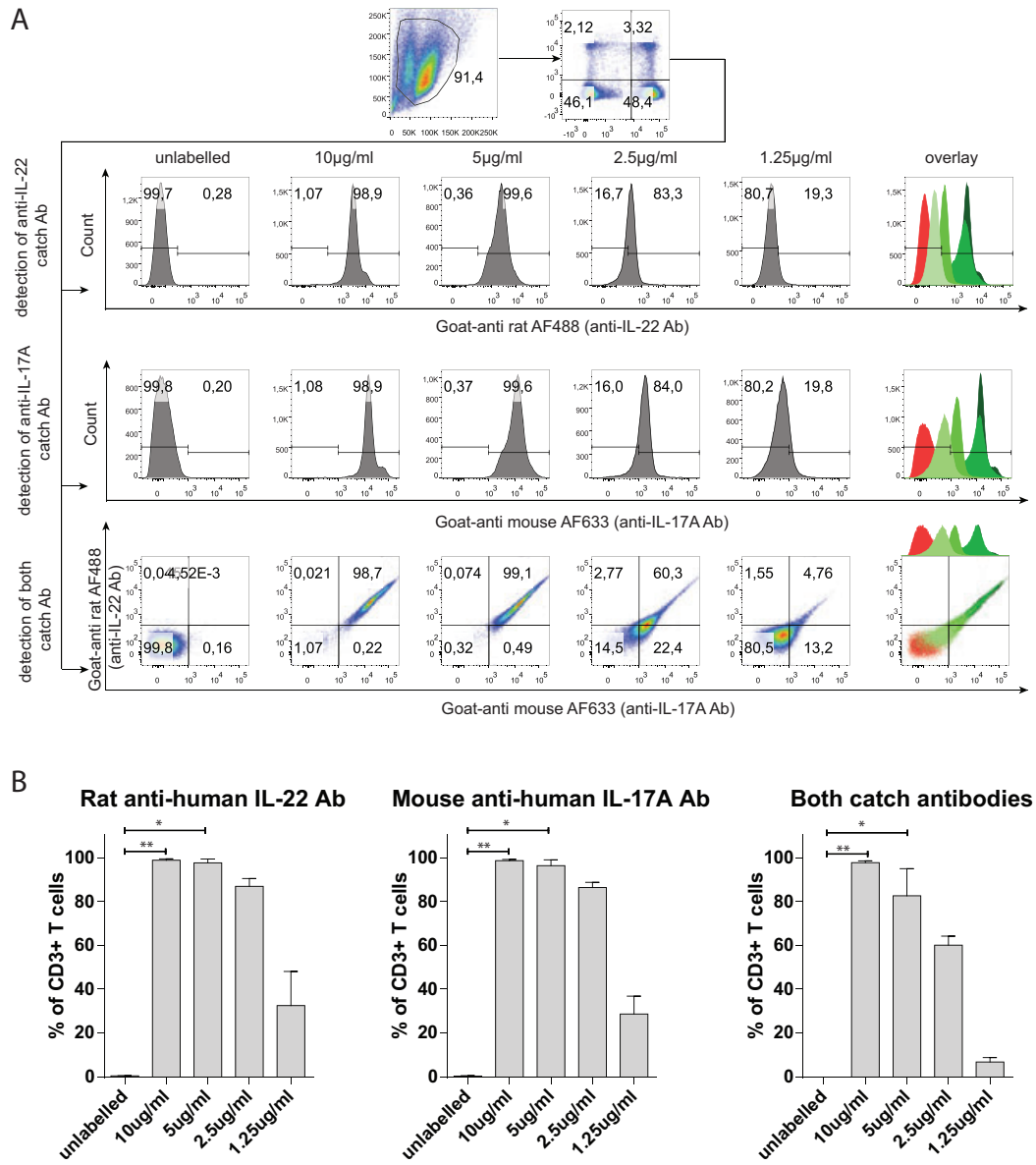


Figure 2.

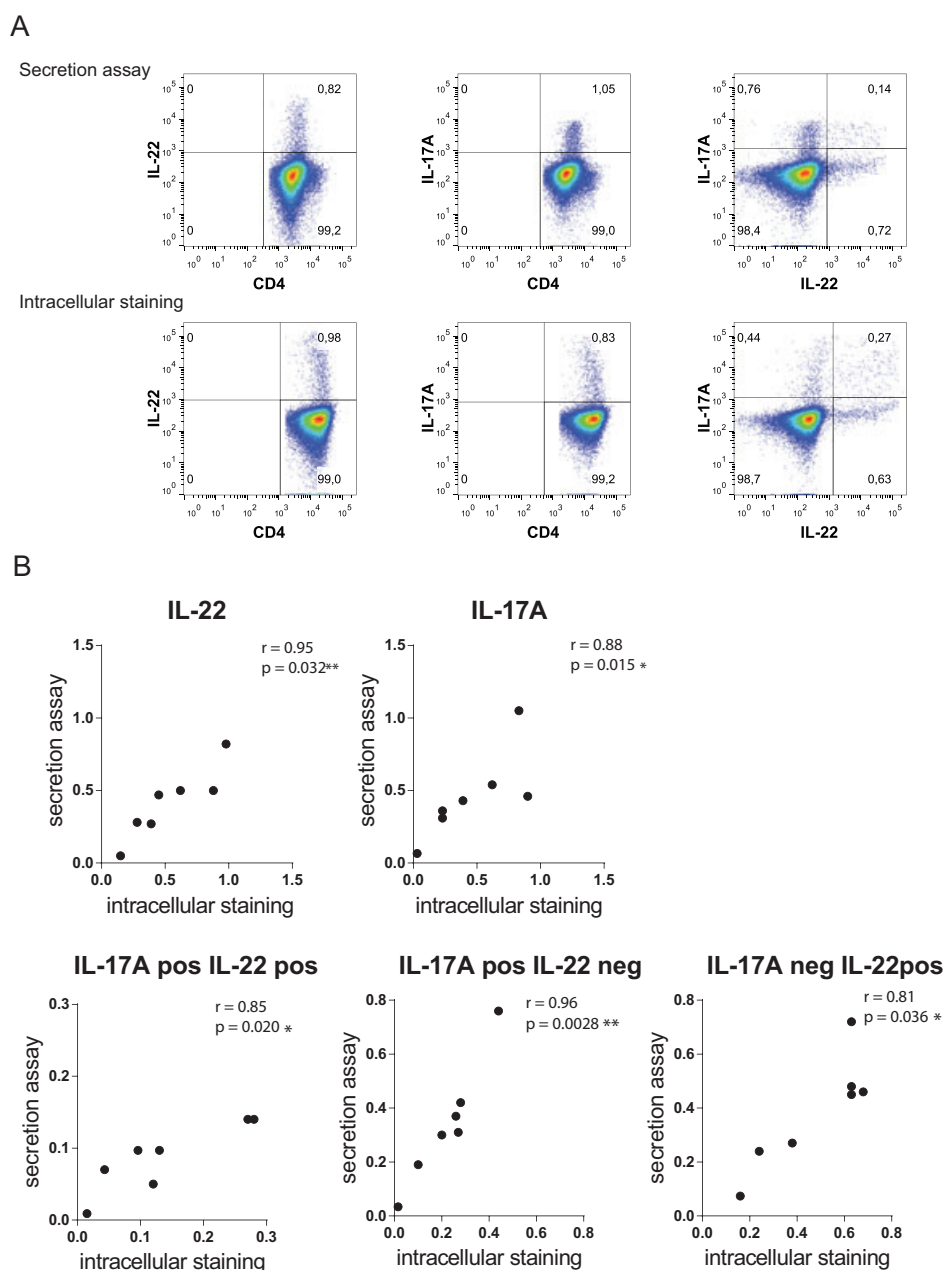


Figure 3.

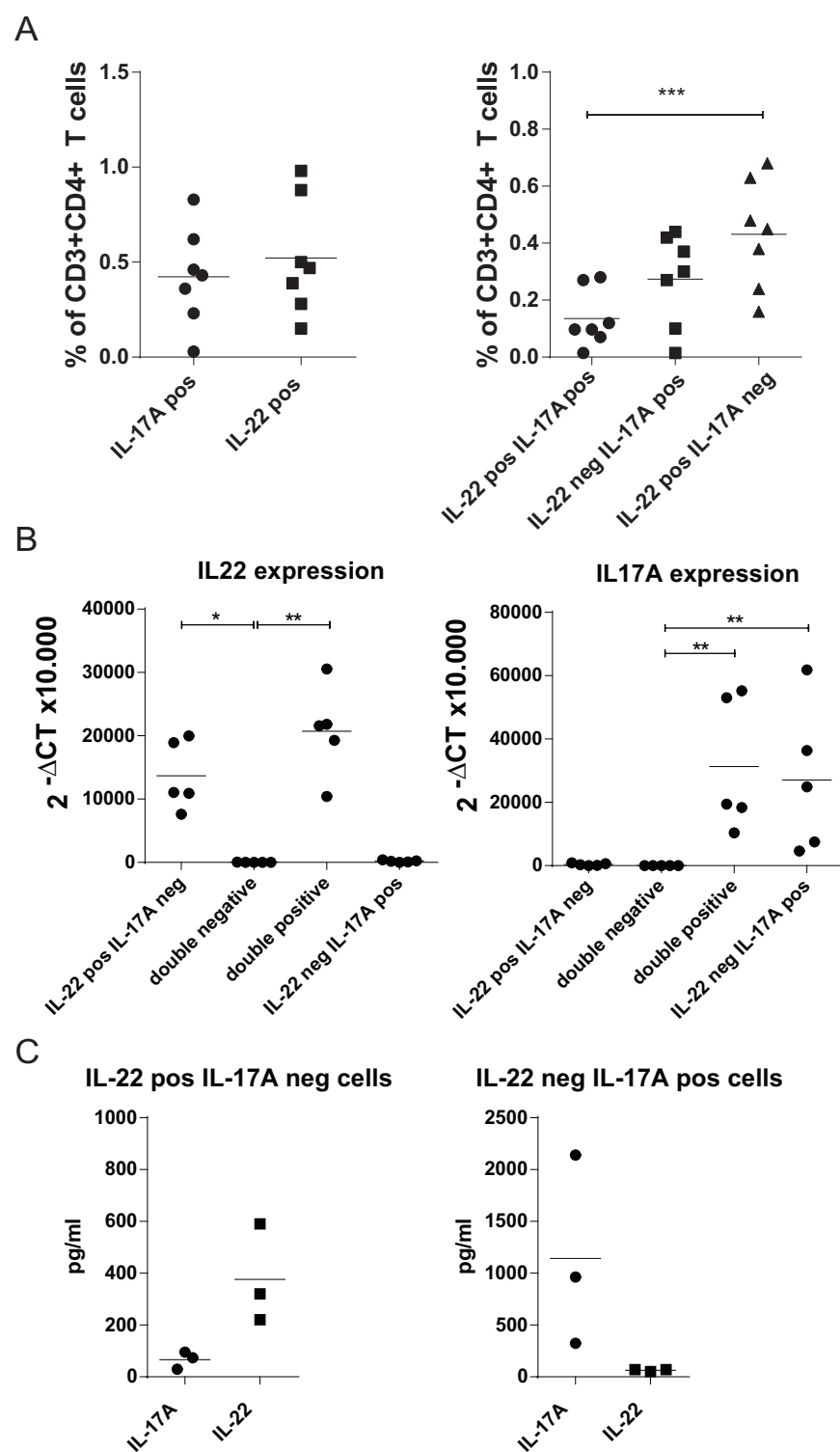
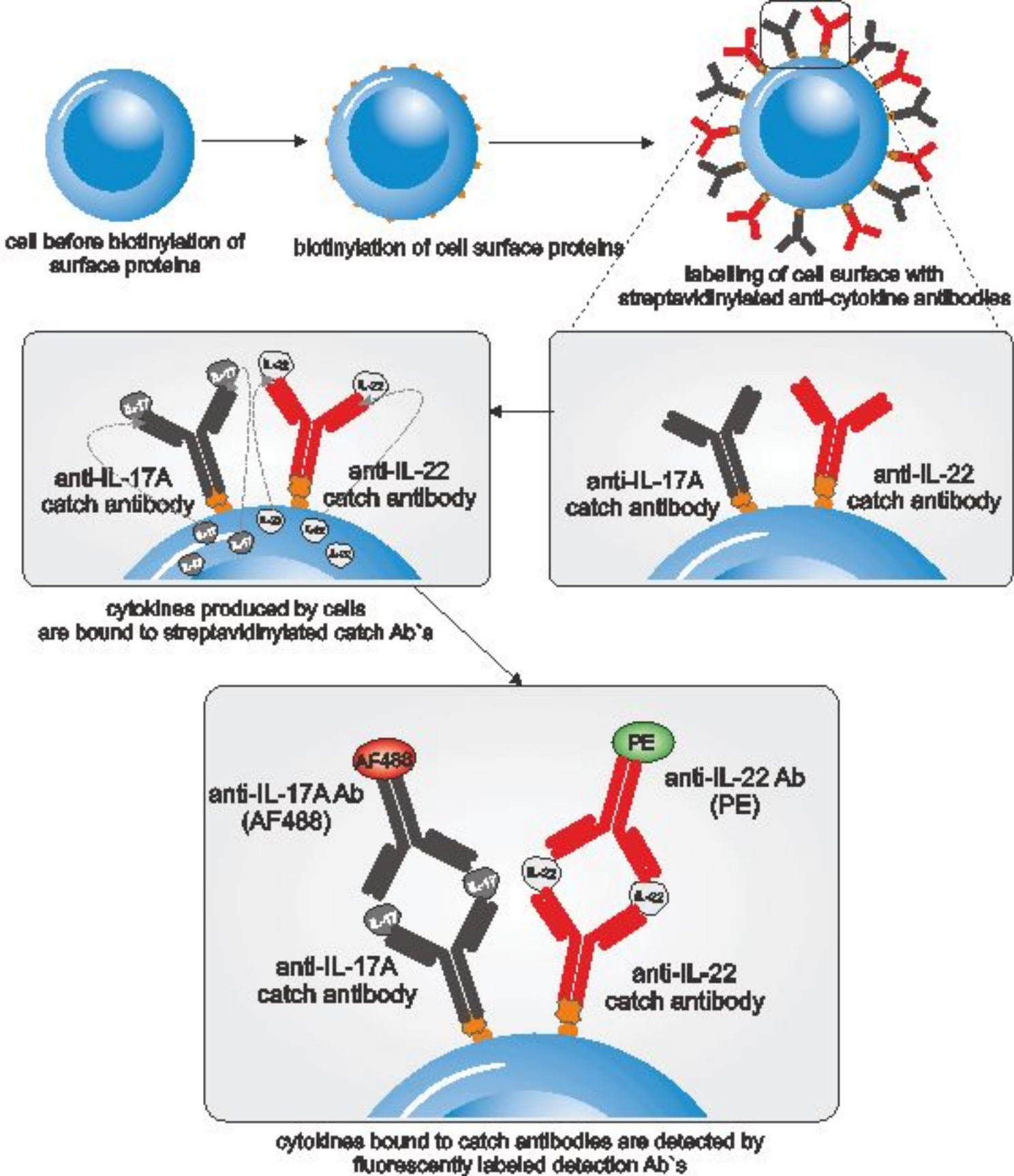
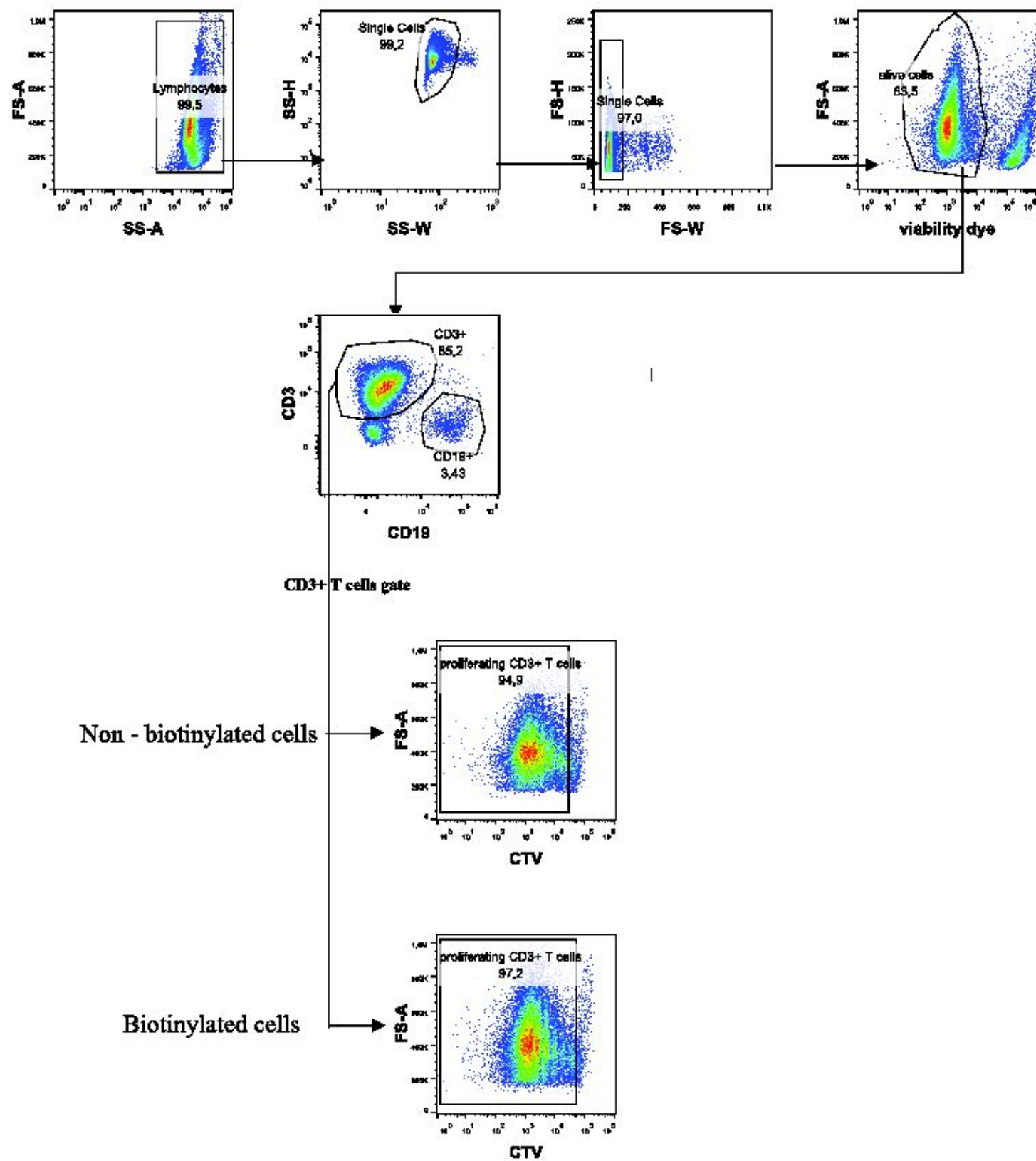


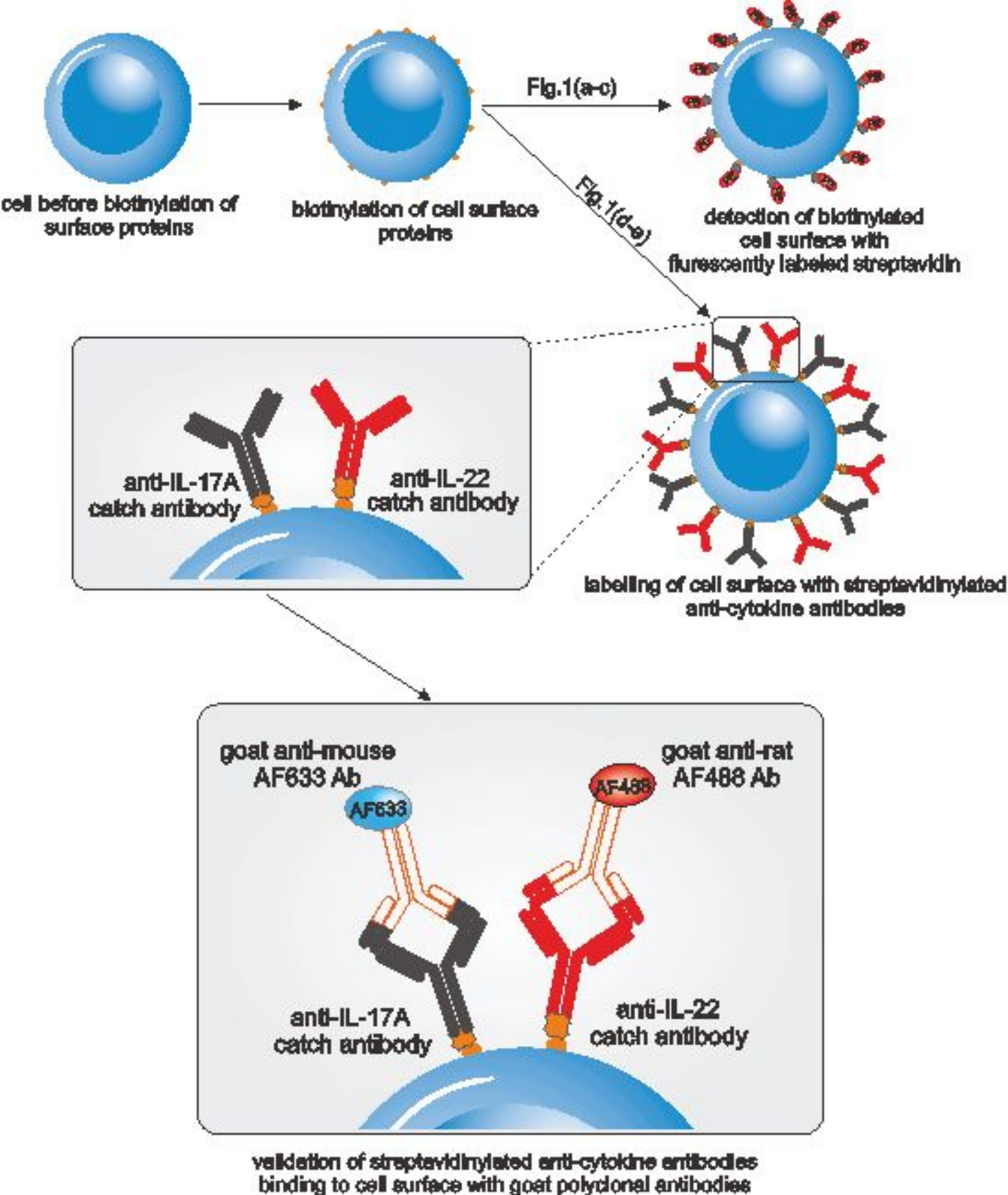
Figure 4.



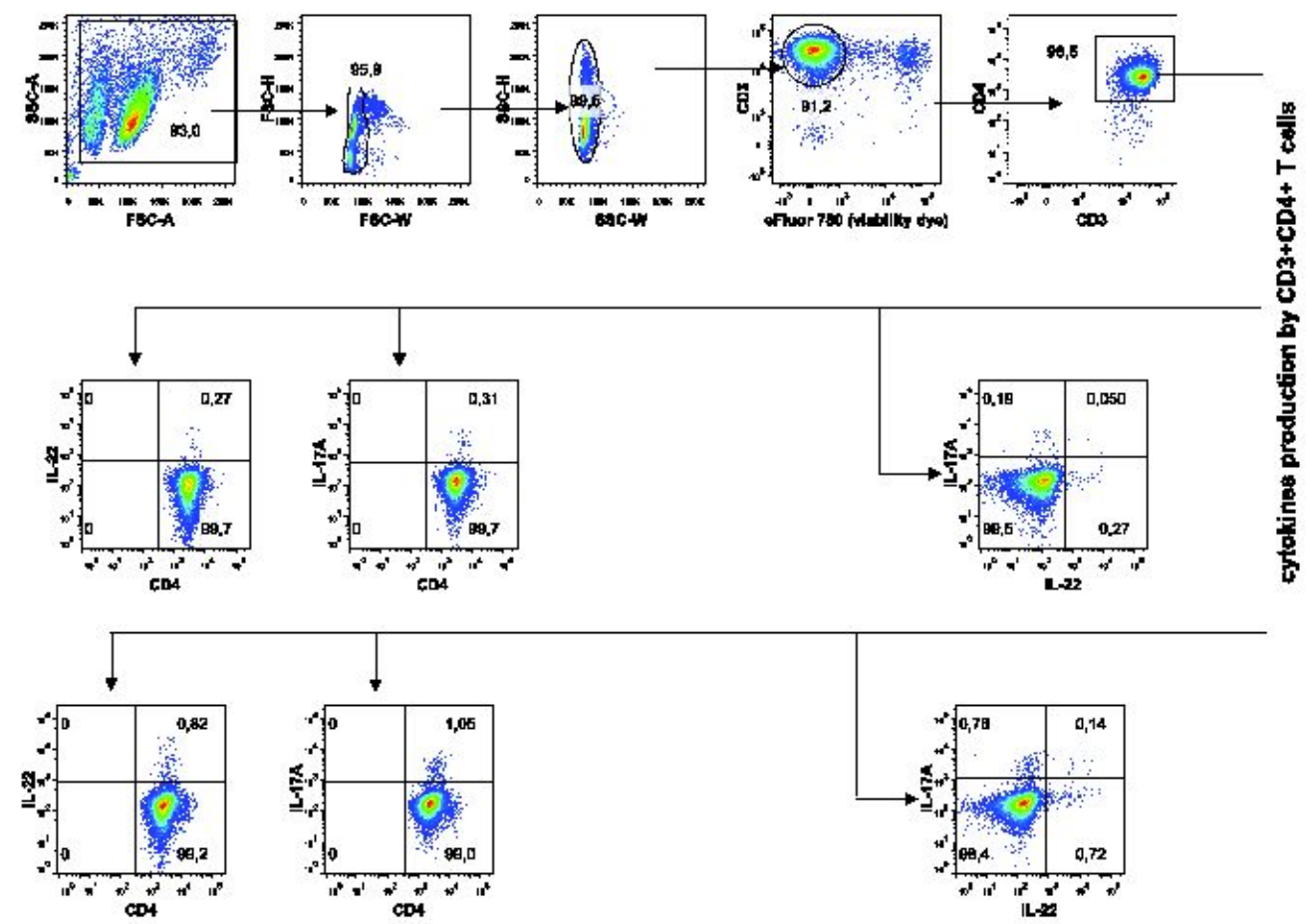
Supplementary Figure 1.



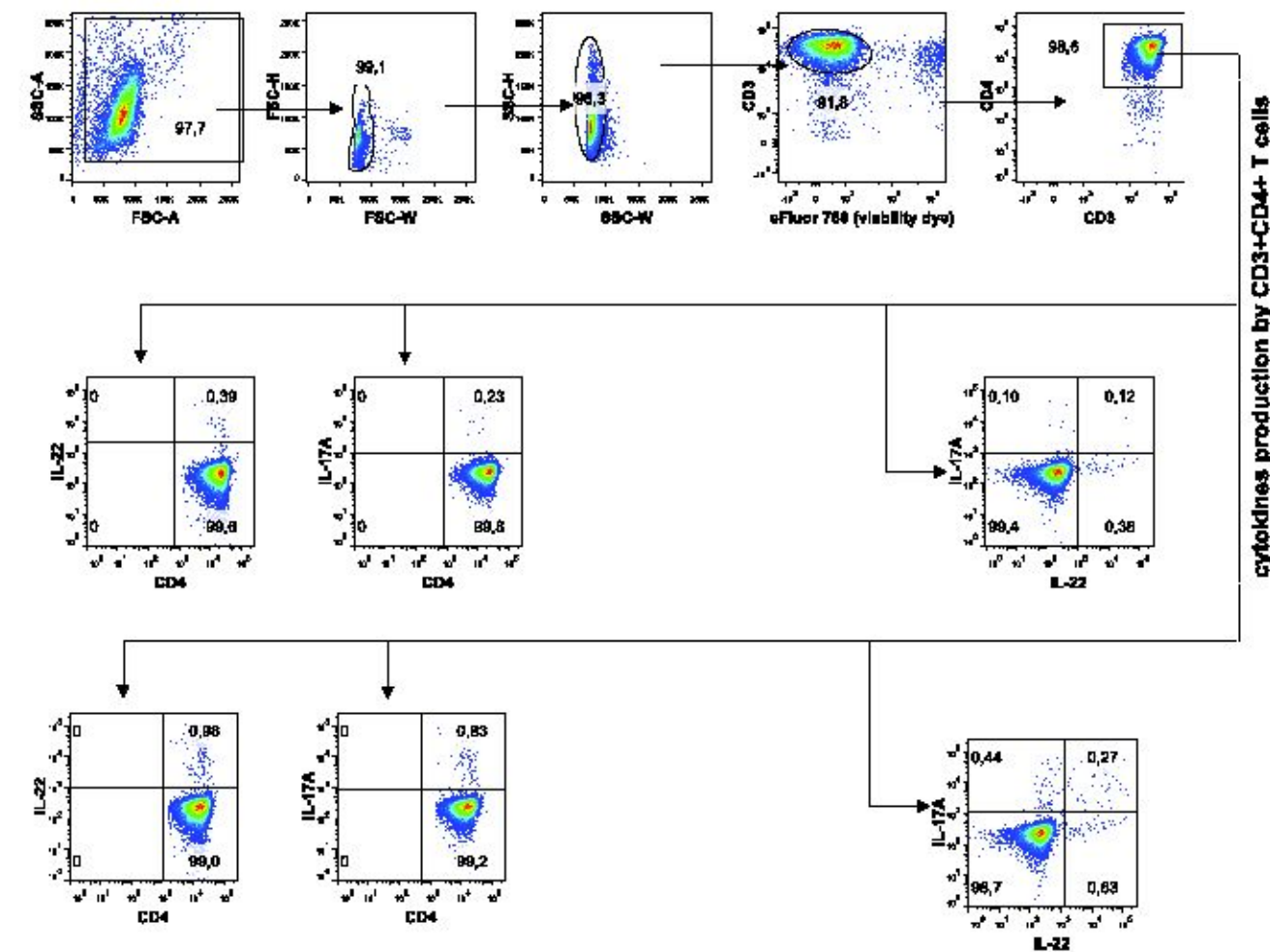
Supplementary Figure 2.



A - secretion assays



B - intracellular staining



Supplementary Figure 4.

1) Labeling cell surface proteins with biotin

- Collect cells from cell culture¹
- Wash cells 3 times with PBS pH 8.0 (300.g, 7 min)
- Divide cells for final concentration of 10×10^6 per tube
- Add 1ml of 1mg/ml biotin solution to each tube
- Mix and incubate for 30 min at room temperature
- Wash cells 3 times with PBS + glycine(100 nM) (300.g, 7 min)

2) Labeling of biotinylated cells with anti-cytokine catch antibodies

- Wash cells with staining buffer* (300.g, 7 min)
- Stain cells with 10 µg/ml of anti-IL-22 and anti-IL-17A catch antibodies, 5µl (0,25ug) of CD3 and 5µl (1ug) of CD4 antibodies premixed together in the final volume of 100 µl staining buffer
 - Include controls, which do not contain anti-cytokine catch antibodies ("no-catch antibody control")
- Incubate cells for 20 min at 4-8°C
- Wash cells with staining buffer (300.g, 7 min)

3) Capture of cytokines secreted from cells by anti-cytokine catch antibodies

- Resuspend cells in pre-warmed at 37°C medium for final concentration of 1×10^6 cells/ml²
- Incubate cells on tube rotator in 37°C incubator for 4 h³
- Wash cells (300.g, 7 min)
- Wash cells with staining buffer (300.g, 7 min)

4) Detection of cytokine producing cells with anti-cytokine fluorescently labeled antibodies

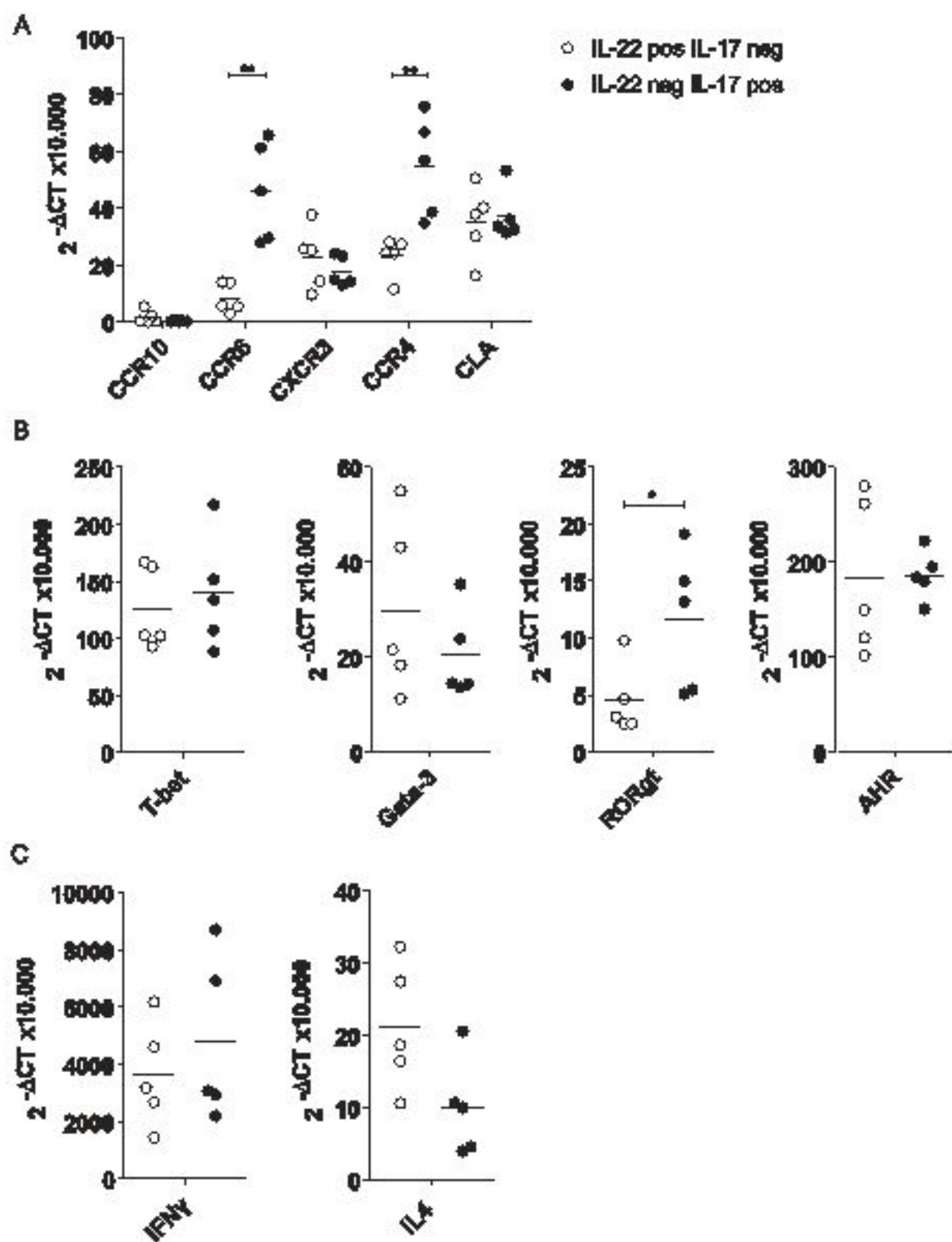
- Stain cells with 10 µl of anti-IL-22PE (12 µg/ml) and 10 µl of anti-IL-17A AF488 (50 µg/ml) antibodies in total volume of 100 µl staining buffer
- Incubate cells for 20 min at 4-8°C
- Wash cells with staining buffer (300.g, 7 min)
- Just before sorting, stain cells with 5µl (0,25ng) of 7AAD viability dye for 5 min
- Resuspend cells in PBS and proceed for FACS sorting of the populations of interest.

¹To reduce amount of reagents used to isolate cytokine producing T helper cells, it is recommended to start whole staining procedure with pre-isolated CD3+CD4+ T cells.

²To minimize probability of non-specific binding of cytokines to catch antibodies on the surface of cells which have not produced cytokines, cells are diluted to concentration 1×10^6 cells/ml medium.

³For the purpose of this presented study cells were stimulated with PMA/Ionomycin to resemble stimulation used in case of intracellular cytokine staining.

* staining buffer contains: PBS + 0.5% BSA + 2mM EDTA



Supplementary figure 6

Supplementary table 1.

Primers sequences used in analysis of phenotypic differences between IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} sorted T cells.

Gene		Primer sequence
<i>CCR10</i>	forward	5` GAGGCCACAGAGCAGGTTTC 3`
	reverse	5` GGGAGACACTGGGTTGGAAG 3`
<i>CCR6</i>	forward	5` GAGAGGGGCCACGTGTATATG 3`
	reverse	5` ATTGATTCCCCGCTCATTGTG 3`
<i>CXCR3</i>	forward	5` GAAGTACGGCCCTGGAAGAC 3`
	reverse	5` CGGCGTCATTTAGCACTTGG 3`
<i>CCR4</i>	forward	5` AAAGCAAGCTGCTTCTGGTTG 3`
	reverse	5` AGGAAGAGCTCCCCAAATGC 3`
<i>CLA</i>	forward	5` GGCTGGGACCTTGTCATAA 3`
	reverse	5` TCAGCAACAGGAGGAGTTGC 3`
<i>T-bet</i>	forward	5` GCAGGGACGGCGGATGTT 3`
	reverse	5` CAAATGAACTTCCTGGCGCA 3`
<i>Gata-3</i>	forward	5` GCGCCGTCTTGATACTTTCAG 3`
	reverse	5` GGGTCACCTGGGTAGCGAAG 3`
<i>Roryc</i>	forward	5` AGCTAGGTGCAGAGCTTCAG 3`
	reverse	5` ATTTGTGTTCTCATGACTGAGCC 3`
<i>AHR</i>	forward	5` TGGTTGTGATGCCAAAGGAAG 3`
	reverse	5` AAGCAGGCGTGCATTAGACT 3`
<i>IL4</i>	forward	5` ACA GCC TCA CAG AGC AGA AGA CT 3`
	reverse	5` GTG TTC TTG GAG GCA GCA AG 3`
<i>IFNγ</i>	forward	5` TCT CGG AAA CGA TGA AAT ATA CAA GTT AT 3`
	reverse	5` GTA ACA GCC AAG AGA ACC CAA AA 3`

Supplementary figure 1. The principle of cytokine secretion assay.

Biotinylated cells were stained with streptavidin-labeled anti-cytokine catch antibodies. Next, cells were kept on the tube rotator in a 37°C incubator and secreted cytokines were bound to the catch antibodies. In the last step, cytokines bound to the catch antibodies were detected by fluorescently labelled anti-cytokines antibodies.

Supplementary figure 2. Gating strategy for assessment of biotinylated and non-biotinylated cells proliferation.

After gating in lymphocytes, doublet cells were excluded and all viable cells were included into analysis. CD3+ T cells were separately gated from CD19+ B cells and their proliferation was analysed. A representative gating strategy shows cells stimulated with anti-CD2, CD3, CD28 mAb's mixture for 6 days.

Supplementary figure 3. Validation of cytokine-secretion assay formation - graphical explanation.

Biotinylation of cell surface proteins was detected with fluorescently labelled streptavidin. In the next step, anti-cytokine catch antibodies were used to stain the cells. To detect the presence of catch antibodies on cell surface, goat anti-rat AF488 antibody and goat anti-mouse AF633 antibody were used to detect rat anti-human IL-22 Ab and mouse anti-human IL-17A Ab, respectively.

Supplementary figure 4. Detailed staining for representative donors in intracellular staining and secretion assays.

Detailed information about percentage of cells obtained from 2 donors (high and low cytokine producers) in secretion assay staining (A) and in intracellular staining (B). Gating strategy includes all cells based on forward scatter (FSC) and side scatter (SSC) and excludes dead cells using eFluor780 viability dye. Next, CD3+CD4+ T cells were investigated for expression of IL-17A, IL-22 or co-expression of both cytokines.

Supplementary figure 5. Protocol for staining IL-22- and IL-17A-producing CD3+CD4+ T cells with cytokine secretion assays.

Detailed protocol of staining, which allows replication of data obtained in the presented paper.

Supplementary figure 6. Phenotypic differences between IL-22^{pos}IL-17^{neg} and IL-22^{neg} IL-17^{pos} sorted T cells.

mRNA expression of chemokine receptors (CCR10, CCR6, CXCR3, CCR4), skin homing marker - CLA, transcription factors (T-bet, Gata-3, RORgt, AHR) and cytokines (IFN-γ, IL-4) was analyzed in IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} sorted T cells. Mann Whitney test was applied (* p<0.05, ** p<0.005).